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Temporal Changes in Routine Health Markers and Their Association with Illness and Mortality Rates in Cats Naturally Infected with Feline Immunodeficiency Virus

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TEMPORAL CHANGES IN ROUTINE HEALTH MARKERS AND THEIR ASSOCIATION WITH ILLNESS AND MORTALITY RATES IN CATS NATIRALLY INFECTED WITH FELINE IMMUNODEFIENCY VIRUS

A Thesis

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by

Jamieson J Nichols

In Partial Fulfillment of the

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of

Master of Science

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Purdue University

West Lafayette, Indiana

FOR “MY” CATS.


Bosco
Benedict
Timothy
Pearl
Flora
George
Manfred
Ernesto
Ted
MrsWootrie
Ace
RaggedyMan
Jimmy
Dakota
Bronks
Gary
Adam
Nigel
Minks
Bertie
CharlieBonney
Baby
BigTunaRusty
FriscoHamilton
Amos
HoneyBluebelle
Nella
Sabre
Roxas
Lorikeet
Alton
RumberoRamses
Booth
Chelsea
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Playa
Mia
FluffyTail
Huckleberry
Tank
MrWonderful
Raven
Jinx
ArtfulDodgerBruce
Isadora
Clyde
Titania
Iko
Obiwan
Bonnie
Mushi

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ABSTRACT

Nichols, Jamieson, J., M.S., Purdue University, December 2015, Temporal Changes in Routine Health Markers and Their Association with Illness and Mortality Rates in Cats Naturally Infected with Feline Immunodeficiency Virus. Major Professor: Lynn Guptill

Feline immunodeficiency virus is an important lentiviral infection in cats. Infection is life-long and results in immune compromise, increased susceptibility to opportunistic infections and early mortality. Infection is commonly referred to in three stages: acute, (asymptomatic) and end stage (symptomatic); although not all cats will progress to end stage disease. Acute infection lasts several months during which cats may have mild, transient anorexia, lethargy, fever, lymphadenomegaly and diarrhea. There is loss of mucosal and peripheral CD4 T cells, expansion of a subset of CD8 T cells, $CD8\beta^{low}$, and establishment of viremia during this period. Asymptomatic infection lasts a variable period of years during which progressive decreases in CD4 T cells and inversion of the CD4:CD8 ratio occurs. End stage infection does not occur in all infected cats. It is characterized by a rapid decline in health including generalized muscle wasting, treatment-refractory opportunistic infection and / or neoplasia leading to death within months. A marked decrease in CD4 T cell counts and CD4:CD8 ratio and several fold increase in viremia occurs during end stage disease but reported literature is sparse. Longitudinal studies of specific pathogen free cats experimentally infected with FIV infection have focused primarily on acute and early asymptomatic stages of infection. Little has been reported on the transition from late chronic to end stage disease. Longitudinal studies of cats naturally infected with FIV are needed to better characterize the course of infection.

In this study, a cohort of cats naturally infected with FIV (n = 87) and uninfected cats (n = 87) were monitored over a period of 3 to 5 years, depending on when they were enrolled. FIV-infected cats were examined twice yearly and uninfected were examined once yearly. At each examination blood samples were collected for routine hematological and biochemical screening

and lymphocyte immunophenotyping. It was hypothesized that FIV-infected cats, compared to FIV-uninfected cats, will have: 1) an increased rate of illness and mortality; 2) differences in temporal patterns for routine hematological and biochemical tests, and lymphocyte immunophenotype; and 3) changes in lymphocyte immunophenotype and clinical pathology markers associated with illness and mortality rates. The same hypotheses were also tested between FIV-infected cats in sanctuary housing and FIV-infected cats living in private homes of <7 cats.

Results showed that mortality rate was increased by 11.7 fold in FIV-infected cats in sanctuary housing and 4.1 fold in FIV-infected cats in private homes compared to FIV-uninfected cats. Illness rates were increased 15.1 fold higher in sanctuary housed cats and 3.0 fold higher in privately homed cats compared to FIV-uninfected cats. FIV-infected cats in sanctuary housing had a 5.1 fold increased mortality rate associated with FIV infection compared to FIV-infected cats living in private homes.

Differences in temporal patterns were statistically significantly different for absolute CD4 T cell counts, total CD8 T cell counts, lymphocytes numbers, albumin, blood urea nitrogen (BUN), and cholesterol levels for FIV-infected cats compared to uninfected cats. Although T cell and lymphocyte counts decreased in both groups, the decreases were greater for FIV-infected cats. Albumin and BUN values decreased and globulin values increased in FIV-infected cats compared to uninfected cats but the changes were small. Differences in $CD8\beta^{low}$ cells counts, CD4:CD8 ratio, globulin and total protein levels were seen but remained stable between the groups over time. FIV-infected cats in sanctuary housing showed statistically significantly different temporal patterns for absolute white blood cell counts (WBC), hematocrit, total protein (TP), BUN, creatinine and alanine aminotransferase (ALT) but the changes were small. WBC and TP values increased in FIV-infected cats in sanctuary housing compared to cats in private homes. Decreasing temporal patterns in BUN, creatinine, ALT and hematocrit were found in FIV-infected cats in sanctuary housing compared to cats in private homes but changes were small and median values remained within the reference intervals. Higher absolute neutrophil counts and globulin levels and lower values for albumin and cholesterol levels were seen in FIV-infected cats in sanctuary housing compared to cats in private homes but differences remained stable between the two groups.

In FIV-infected cats, a greater decrease in CD4 T cell and albumin percentages and a greater increase in CD4:CD8 ratios and neutrophil counts were associated with an increase in illness rate. An increase in mortality rate was associated with a greater increase in total protein and greater decreased in albumin and hematocrit percentages. However, associations were weak and use of any of these parameters as a stand-alone monitoring tool cannot be recommended. There were no associations between any parameter and illness or mortality in FIV-uninfected cats.

Results of this study showed FIV-infected cats had increased illness and mortality rates compared to FIV-uninfected cats. Furthermore, FIV-infected cats living in sanctuary housing had higher illness and mortality rates compared to FIV-infected cats living in private housing. This raises the question of what role environmental conditions may play in disease progression in FIV infection. This has not been previously reported in the literature and further investigation into the potential environmental impact on disease progression is warranted.

The most notable differences in temporal patterns for health parameters were associated with inflammation and immune cells. Illness and mortality rates were associated with greater changes in inflammatory parameters and lymphocyte immunophenotype in FIV-infected cats, although, associations were weak. While considered exploratory in this study population, these findings further support the roles of chronic inflammation and immune activation in disease progression following FIV infection that have been reported in the literature. Given the limited availability of monitoring techniques for FIV-infected cats, further investigation into the use of these health markers of inflammation and lymphocyte immunophenotype as a panel to monitor disease progression is warranted. However, continued research into new, reliable and easily performed techniques that accurately measure chronic inflammation and immune activity in FIV infection is needed.

CHAPTER 1. INTRODUCTION

Feline immunodeficiency virus (FIV) is an important worldwide lentiviral disease in cats similar to human immunodeficiency (HIV) and simian immunodeficiency virus (SIV). Worldwide disease prevalence has been reported between 2 and 34% depending on location, living environment and health of the cat[1,2]. FIV is a complicated lifelong disease that results in immunocompromise and a chronic inflammatory state within the host. Infection is most commonly spread through deep, penetrating bite wounds and male, intact, outdoor cats are considered at highest risk due to territorial fighting behavior[3,4].

Following infection there are 3 stages of infection: acute, chronic (asymptomatic) and end stage (symptomatic). In the acute stage of infection, cats may experience transient clinical signs of illness including fever, lethargy, anorexia and diarrhea beginning approximately 6-8 weeks post-infection[5]. Mucosal and peripheral blood CD4 T cell populations are greatly reduced, $CD8\beta^{low}$ T cell populations are expanded and viremia is established[5-8]. The expansion of $CD8\beta^{low}$ cells appears to be unique to FIV infection and studies have shown that as this population of cells is expanded viremia is decreased[9]. However, infected cats are unable to clear the virus resulting in life-long infection and persistent low level viremia[10,11]. Once viremia is brought under control, CD4 T cell counts recover but typically not to pre-infection levels and cats transitions into a chronic asymptomatic phase of infection which lasts a variable period of years. During this time, cats have no detectable abnormalities on physical examination. There is, however, a gradual decrease in CD4 T cell counts, expansion of $CD8\beta^{low}$ cells, progressive inversion of the CD4:CD8 ratio, and an undulating viral burden which at times is below the limit of detection[10,12]. Progressive immune alteration eventually results in immunocompromise and may increase susceptibility to certain infections and neoplasias. It is not known what proportion of cats will progress to end stage infection. However, it is known that once in the end stage of infection, decline in health is rapid, typically over several months, and death is imminent. Of cats that do

progress to end stage infection, treatment refractory opportunistic infections, generalized muscle wasting, chronic gastrointestinal disease and neoplasia are common[13,14]. It is thought that cats in end stage infection have a resurgence of viremia, extreme loss of CD4 T cells, and marked anemia and leukopenia, however, this has not been extensively studied[15-17].

Several underlying mechanisms of immunocompromise and chronic inflammation are similar between FIV, HIV and SIV; although mechanisms in HIV have been more intensively studied and reported. For example, in FIV-infected cats, cytokine dysregulation, increased T cell anergy and apoptosis, and increased regulatory T cell (T_{reg}) activity have been associated with FIV disease progression[18]. Altered cytokine production resulting in increased circulating pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1, IL-10, and tumor necrosis factor alpha (TNF-α) and decreased IL-12; favors humoral (antibody) immunity and limits cell-mediated immunity, leaving infected cats unable to mount an appropriate immune response to intracellular pathogens[19-22]. T_{reg} cell activation limits the amount of IL-2 produced; a key cytokine produced by T cells to propagate an adaptive immune response to pathogens[23]. Up-regulation and overexpression of several surface proteins on activated T cells, namely CTLA-4 and B7.1, act as co-stimulatory signals between T cells and induce a state of anergy (immune inactivity)[24,25]. These factors may create a paradoxical situation, in which, immune activation stimulates dampening of the adaptive immune response, rather than proliferation of a protective response. Similar activity has been reported in HIV and SIV.

The body of knowledge regarding the immune response following FIV infection has been gleaned from experimental studies. Experimental longitudinal studies tend to focus primarily on acute and early chronic infection[5,6,21], with limited studies on the transition from chronic to end stage infection[15,16]. In contrast, studies of natural infection tend to be cross-sectional studies that reported seroprevalence and / or mean differences in health markers between FIV-infected and uninfected cats at one point in time [3,26,27]. Several factors complicate long term studies of FIV, whether experimentally or naturally acquired, including the long duration of infection, uncertainty of which cats will progress to end stage infection, lack of known factors that underlie disease progression, and the expense associated with monitoring cats over a prolonged period of time. Some investigators have expressed concern that experimentally induced infection may not accurately mimic naturally acquired infection[10]. Infecting dose is unknown, as is the impact of different inoculating routes, and pathogen exposure disparity between specific pathogen free

housing of experimental settings and the variety of housing conditions for privately owned and shelter housed cats.

To date there are no readily available monitoring methods for characterizing immune system function changes or for predicting which FIV-infected cats will progress to end stage disease and when. Current monitoring guidelines for FIV-infected cats include twice yearly physical examinations and once yearly routine hematological and biochemical screenings[28,29]. While important, these screenings only report hematological or biochemical abnormalities and do not evaluate immune activation or inflammatory mediators.

The study reported here monitored a cohort of naturally FIV-infected cats living in either sanctuary housing or private homes and FIV-uninfected cats over a period of up to 5 years. During the study period, FIV-infected cars were examined twice yearly and FIV-uninfected cats were examined once yearly. At each examination blood and urine was collected for routine hematological and biochemical screening as well as lymphocyte immunophenotyping. Physical examination and laboratory data were assessed and used to calculate illness and mortality rates, assess for differences in temporal patterns for laboratory parameters, and for association of inflammatory, immunophenotype and hepatic markers with illness and mortality in FIV-infected and FIV-uninfected cats.

The study was designed to address the following hypotheses: FIV-infected cats, compared to FIV-uninfected cats will have: 1) higher illness and mortality rates; 2) differences in temporal patterns of change CD4 and total CD8 lymphocyte counts and ratios, and hematological and biochemical parameters; and 3) changes in markers of inflammation, lymphocyte immunophenotype associated with illness and mortality rates over the 1 year interval prior to progression to illness or death. These hypotheses were also examined for FIV-infected cats in sanctuary housing compared to FIV-infected cats in private homes of <7 cats.

The information gained from this study will be important for privately owned cats, and also for FIV-infected shelter housed cats. These cats represent an emerging population as animal sheltering systems move toward a model where FIV-infected cats are placed for adoption rather than euthanized. Cats housed in shelters live within a dynamic housing environment and face higher exposure to pathogens than do cats living in private homes with smaller numbers of cats. For FIV-infected cats, the shelter environment, with ongoing exposure to new cats and pathogens, places further stress on the FIV-altered immune system. Without other readily available

diagnostics to assess disease progression in FIV-infected cats, identification of easily monitored markers associated with illness and mortality is important for maintaining the health of these cats. In a broader scope, these findings may help guide future husbandry and management strategies to maximize health in FIV-infected cats, particularly shelter housed cats where improved knowledge of health status may indirectly improve adoption rates of these cats.

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CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Feline immunodeficiency virus (FIV) is an important infectious disease affecting cats worldwide. The seroprevalence of antibodies to FIV infection in various worldwide cat populations varies and has been reported to be between 2% and 34%[1-14]. Seroprevalence is higher in adult, male cats with outdoor access and has been reported to be higher in cats presented to veterinarians with clinical signs of illness than in healthy cats[6,13-15]. The virus is most commonly spread through deep, penetrating bite wounds and the higher infection prevalence in intact, outdoor, male cats is thought to be due to territorial fighting behavior.

Similar to human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), the FIV course of infection can generally be divided into an acute phase of several months, a variable period of chronic asymptomatic infection lasting a variable number of years and finally progression to severe immunodeficiency and end stage disease[16-19]. The viruses cause chronic immune activation and impaired response to infection, with immune compromise and eventually poor health. There is progressive loss of circulating CD4 T cells leading to persistent inversion of the CD4:CD8 ratio[20-24], and loss of structural immune integrity including disruption of the intestinal barrier and depletion of mucosal immune cells[25-31]. As a result of progressive immune compromise during the chronic phase of infection, patients are at risk for developing certain diseases and opportunistic infections such as neoplasia, periodontal disease, chronic gingivostomatitis, and upper respiratory infections[18,19,32]. In the early symptomatic stage of infection oral, respiratory, dermal and intestinal infections are common but typically respond to medical management and care. However, progression to end stage immunodeficiency often leads to treatment-refractory opportunistic infections, wasting, anemia, leukopenia, neoplasia, and death[17,24,33]. In humans, CD4 T cell counts and viral load are routinely monitored to assess the need for medical intervention with anti-retroviral therapies (ART) and to evaluate response to

ART[34,35]. However, in some HIV-infected individuals, there is progression to end stage disease despite vigilant monitoring and positive response to ART therapies[36,37]. It has been reported that progression of disease in such individuals may be related to chronic immune activation[38], and that microbial translocation from the intestinal lumen to systemic circulation is an underlying cause of chronic immune activation[26,39]. Patients evaluated had bacterial 16S rRNA, lipopolysaccharide (LPS), and increased lipopolysaccharide binding protein (LBP) in circulation as well as increased serum concentrations of the inflammatory cytokines interleukin-1β (IL-1β), IL-6, soluble CD14 (sCD14), and tumor necrosis factor alpha (TNF-α), supporting the hypothesis that microbial translocation is associated with chronic immune activation and HOV disease progression[26,40]. As a result, these cytokine biomarkers are being considered for routine monitoring of HIV-infected persons to assess and predict disease progression.

Monitoring of CD4 T cell counts and viral loads is not readily available for use in FIV-infected cats and ART therapies are not widely recommended due to serious adverse effects[41-45]. Recommendations for monitoring FIV-infected cats currently include twice yearly physical examinations and yearly routine hematological and serum biochemistry tests[46-48]. However, these measures only identify progressive disease rather than predict progression. Given the similarities between HIV and FIV, similar monitoring techniques may allow identification of immune activation and prediction of progression to clinical disease in FIV-infected cats. This could result in earlier medical intervention to prevent or slow progression to symptomatic infection and extend good quality of life. As the shelter industry moves to adopt rather than euthanize FIV-infected cats, there is an emerging population of FIV-infected cats in shelters. Monitoring techniques that can assess immune health and predict progression to symptomatic infection may help improve the care and management of FIV-infected shelter cats and improve adoption rates by providing a better health assessment to potential adopters.

This review provides a general overview of epidemiology, transmission, diagnosis and course of FIV infection. It addresses mechanisms of cellular infection, virus dissemination and immune impairment including histopathologic alternations secondary to infection and compares FIV to human immunodeficiency virus (HIV). A summary of future directions for patient monitoring and therapy, which may be applicable to future directions in FIV research, is also presented.

2.2 Feline Immunodeficiency Virus

Feline immunodeficiency virus is a lentivirus in the family Retroviridae. This group of viruses uses reverse transcriptase to make a double stranded DNA (dsDNA) copy of viral RNA and insert it into the host cell genome. Insertion into the host genome allows for transmission of the virus to daughter cells during cell division and propagates life-long infection[49,50]. Lentiviruses, including FIV, HIV and SIV, have similar genes including *env*, *gag* and *pol*[49]. The proteins encoded by these genes have specific functions that allow for replication and survival within the host. The *env* gene encodes for envelope proteins associated with attachment, fusion and infection of target cells. This region is the most variable and is prone to mutation or recombination events that help the virus evade the immune system in circulation. The *gag* gene encodes the structural proteins of the virus and is less prone to mutation and recombination. The *pol* gene encodes the transcriptional proteins, most notably reverse transcriptase, and is the most conserved allowing for successful replication and integration of new viral DNA into the host genome[49,50].

Several FIV subtypes exist based on phylogenetic mapping of these three genetic regions and are identified as A through F[51-56]. FIV has a worldwide distribution and the most prevalent subtype varies by location. Subtypes A and B have the broadest worldwide distribution. Subtype C is rare in the United States but prevalent in Australia and Canada[57,58]. Subtype D is reported in Japan and Vietnam, and subtype E is found in Argentina[55,56]. Subtype F, thought to be a variant of subtype B, has only been identified in the United States[51,52]. Based largely on experimental infection, using differing strains and infecting doses of FIV in kittens and cats of varying ages, there appear to be differences in subtype pathogenicity. These experimental observations on subtype pathogenicity have not been reported in naturally acquired FIV infection of domestic cats. In general, subtype B is believed to be the least pathogenic, resulting in the mildest clinical signs and longest time to disease progression[54]. This subtype is also more genetically diverse and may show the most host adaptation based on studies of wild cat populations[54,59]. Subtype C has been reported as the most virulent and pathogenic strain resulting in rapid disease progression and death[60]. Subtype A has moderate pathogenicity and may be more associated with the development of neurological disease signs than other subtypes[54].

Commonly, subtyping of FIV has been performed using the variable regions 3 through 5 (V3-V5) of the *env* genes however, use of the *gag* and *pol* genes has also been performed and similar subtyping results are reported between genes[57]. Diversity within the V3-V5 *env* regions of subtypes A, B and C has been reported to be 3-15% within a subtype and 18-26% between

subtypes[53]. The *env* gene encodes for the outer surface glycoprotein of the virus and is responsible for attachment and entry of the virus into the cell; therefore it is easily detected by immune surveillance cells and serves as the primary target for antibody production. As a result, the *env* gene is under pressure to evade the immune system. One such protective mechanism is recombination which results from crossover between viral genomic RNA regions within the host[57]. Recombinant events between 2 strains of the same subtype can occur as either intragenic or intergenic recombination[57]. Intragenic recombination results from crossover between 2 single gene regions whereas intergenic recombination results from crossover between 2 different gene regions[57]. In a recent study, recombination events in the *env* region occurred in 41% of the naturally infected cats that were studied[61]. In a separate but related study, an evolutionary rate of 1.16×10^{-3} substitutions per site per year was reported in naturally FIV-infected cats, which is faster than $3.1\text{-}6.6 \times 10^{-3}$ rates previously reported[57,62,63]. The rate of viral evolution was found to be higher in those cats without physical examination abnormalities as compared to those without which suggests increased pressure for viral evasion of the immune system in healthy FIV-infected cats[62].

Recombination may also occur when 2 different parent viral strains are present within the host either due to co-infection or super-infection[57,64]. Co-infection, as defined for HIV, results from recent infection with a second viral strain before the first viral strain infection has been established and an immune response developed. Super-infection results when the second viral strain infection occurs after the first viral strain infection was established and an immune response developed[64]. The existence of co-infection and super-infection, in both HIV and FIV, can be difficult to determine in natural infection but the occurrence of phenotypes displaying sequences of multiple subtypes has been reported. In FIV infection, recombination events between A/B, B/D and A/C subtypes have been reported[54,65,66].

2.3 Epidemiology and Transmission

Seroprevalence for FIV antibodies in various countries has been reported to be between 2% and 34%[1-14]. Variability in seroprevalence is attributed to several factors such as whether cats studied are owned, stray or feral, are allowed to roam outdoors or display clinical signs of illness at the time of testing. A recent study in Germany reported the seroprevalence of FIV to be 3.2% in a group of healthy privately owned cats brought to the veterinarian for routine healthcare[6]. A study in Egypt reported the seroprevalence of FIV to be 33.9% in a group of feral cats captured as part of a trap-neuter-release effort[2]. Two other studies, from Japan and Malaysia, reported the

seroprevalence of FIV in healthy cats, with access to the outdoors, to be 4.2% and 23.6%, respectively[3,9]. The latter two studies reported a higher seroprevalence of FIV (18.8% and 38.4%, respectively) in cats identified as unhealthy at the time of testing[3,9]. In the United States and Canada, the overall seroprevalence of FIV is estimated to be approximately 2.5% and 5.5%, respectively, in domestic cats. A higher prevalence has been reported in cats allowed outdoors (4.3%), cats in large sanctuaries (8.8%) and cats presented to veterinarians with clinical signs of illness (up to 20.6%)[14,15,67].

Transmission is reported to occur most commonly through saliva exposure via deep penetrating bite wounds. Adult, intact, outdoor male cats are reported to be at increased risk due to territorial fighting behavior[1,3,13,14,68,69]. There was no reported horizontal transmission of infection in households where FIV-infected and FIV-uninfected cats comingle, are kept strictly indoors, not introduced to new cats, and have minimal inter-cat aggression[3,6,70,71]. Parenteral injection (intravenous or intraperitoneal) of FIV-contaminated tissue establishes infection, however, this is considered an unlikely route of naturally acquired infection[46,72-76]. Transmission during mating appears possible as virus has been detected in feline seminal fluid but this is not thought to be a common transmission pathway[72-76].

Transmission from queen to kitten is reported in experimental infection and infection rates may be as high as 70% but this appears to be uncommon in naturally acquired infection[77]. In experimental settings, transmission from queen to kittens can occur in utero, during parturition with exposure to vaginal secretions and via colostrum and milk[78-81]. FIV-infected queens have a higher rate of abortion, stillbirth and nonviable kitten delivery[78,82]. The rate of transmission during pregnancy seems to be higher in FIV-naïve queens infected during pregnancy and chronically FIV-infected queens with low CD4 T cell counts (<200cells/uL)[79]. Kittens born to FIV-infected queens may test FIV-positive by virus isolation (VI) and polymerase chain reaction (PCR) at birth and by antibody titer as early as 2 weeks of age[78,82]. In experimental settings, some kittens have shown evidence of regressive infection after testing FIV-positive by VI, PCR and antibody test[82]. In regressive infection, there is a loss of detectable FIV antibody, virus and provirus in peripheral blood despite having tested positive at earlier time points. However it is not known if these kittens have truly cleared the virus or if disease remains in tissues at an undetectable level or latent state[78,82]. Similar events have been reported for HIV in perinatally infected children but again, it is not known whether the virus remains in the tissues or has been cleared from the body[83].

2.4 Cellular Infection and Dissemination

2.4.1 Cellular Infection

In FIV, similar to HIV and SIV, CD4 T cells are the primary target for infection during acute infection and throughout the course of disease. The CD4 T cells are a key component of the adaptive immune system and function to direct the humoral (antibody production) and cell mediated (cell-to-cell interactions) arms of the immune system[84]. During the chronic phase of infection, FIV infects macrophages, neutrophils and dendritic cells within the innate immune system. These cells are considered generalized surveillance cells, are the first line of defense against infection, and activate the adaptive immune response.

Similar to HIV, FIV requires a co-stimulatory signal in order to infect an individual cell. FIV utilizes CD134 and CXCR4 receptors on T cells for cell entry[32,49]. The FIV glycoprotein 120 subunit (gp120), encoded on the V3 loop of *env*, first binds to the CD134 receptor on the activated host T cell. This induces a conformational change exposing a cryptic epitope in the V3 loop to allow binding to the CXCR4 receptor, which is the necessary co-stimulatory signal to allow the virus to gain entry into the cell[32,49,85]. Once this attachment is complete the virus fuses with the host cell membrane and the virus particle is brought into the cell.

CD134 is expressed at higher concentrations on activated lymphocytes, in particular CD4 T cells, which are the primary target cell for FIV infection[85]. CD134 is also found on activated CD8 T cells, B cells and monocytes/macrophages[85]. The chemokine, CXCR4, is found on all cells but is expressed in higher concentration on monocytes and macrophages. These receptor location and concentration distinctions help explain the pattern of cellular infection. Host CD4 T cells are the primary targets but as chronic infection is established the target cell population expands to include CD8 T cells, B cells and monocytes. In one study, the shift in cellular infection from CD4 T cells to monocytes and macrophages occurred with the appearance of clinical signs of disease[86]. This shift in cellular infection was thought to reflect the emergence of a viral variant that preferentially infected monocytes and macrophages[87]. Mutations in the *env* region of the virus during replication during experimental infection have been reported to produce a variant that is CD134-independent with an increased ability to infect cells lacking CD134 but expressing CXCR4 (i.e. monocytes and macrophages)[85]. The use of CD134 as a primary receptor for viral entry into a cell involves a complex interaction with the cysteine rich domains 1 and 2 (CRD1 and CRD2) of the receptor but as infection progresses the interaction with the CRD2 region decreases and becomes less complex[88]. It has been suggested that in “early” FIV infection the

virus is CD134-dependent whereas in “late” infection the virus is CD134-independent. It has been reported that removal of a single glycosylation site in the V1-V2 viral homolog contributes to the shift away from CD134 dependence for viral entry[89]. Recently it was reported that a higher proportion of CD134-independent viral variants was associated with an increase in abnormalities on physical examination, CD4 T cell counts <350 cells/μL, and death suggesting that a shift towards these viral variants are associated with disease progression[88].

Once inside the cell, the FIV virus makes a double stranded DNA (dsDNA) copy of its viral RNA, utilizing reverse transcriptase (RT). Creation of dsDNA from viral RNA involves a series of events: 1) RT creates a strand of complementary DNA (cDNA) of the RNA genome, 2) RNase H activity removes the RNA strand from the cDNA strand, 3) the cDNA anneals with the end of another viral RNA strand and polymerization of DNA continues, 4) the annealed RNA is degraded in multiple areas by RNase H, 5) another strand of DNA is created using the remaining segments of RNA as a primer, 6) the DNA strands are separated and 7) the newly formed DNA strand anneals to a different DNA strand to create a complete double stranded DNA (dsDNA). Once complete, this dsDNA copy is inserted into the host genome in the nucleus of the cell.

The dsDNA is transported to the nucleus as part of the pre-integration complex (PIC) and interacts with FIV integrase to tether it to the host cell chromatin. Integration of the dsDNA occurs through cleavage, repair and ligation of the new dsDNA to the host target DNA to create a new genome sequence[49,90]. The insertion of the dsDNA copy into the host genome ensures the viral material will be passed to daughter cells resulting in life-long infection. It also allows evasion of the immune system by disguising itself as part of the host cell genetic material[49,90].

Transcription of viral RNA copies within the nucleus and packaging of new virion particles within the cytoplasm is also performed. New virion particles are released to spread infection to new cells. It is thought that the accessory protein, OrfA, initiates transcription indirectly within the nucleus. Then Rev protein interacts with Rev-response element, both encoded within the virus, to shuttle target RNA out of the nucleus. Once outside the nucleus, proteins are translated from the genomic transcript and then identified and captured for packaging the *gag* proteins. The *gag* proteins provide the main structure of the virion and play a role in budding and release of the virion from cells, although the mechanism is not well understood. Tetherin, a transmembrane protein, anchors the virion to the cell and is thought to interfere with its release, however, with the CD134-independent strain of FIV, tetherin may actually enhance cell-to-cell spread of the virus

by syncytial formation. During maturation of the virion, viral protease cleaves several precursor polyproteins from *Gag* and *Gag-Pol*. These precursors are then further cleaved in specific order to form the mature virion and activate enzymes associated with its ability to infect a new cell. The release of new viral particles contributes to continued infection of naïve CD4 T cells, macrophages and dendritic cells and perpetuates a state of active infection[49,50, 90].

2.4.2 Dissemination

Rapid and widespread dissemination of FIV occurs concurrently with the cellular alterations in peripheral blood and other tissues following experimental infection[87]. Proviral DNA can be detected in 1/1,000,000 circulating PBMC by day 5 post-infection and in 1/100,000 cells day 84 post-infection[87]. Proviral DNA was isolated as early as 10 days post-infection from thymus, lymph nodes, tonsils, lungs and brain and several weeks later from peripheral lymph nodes, spleen, bone marrow, intestines and spinal cord[87]. Viral RNA was detected in PBMC as early as 14 days post-infection at a rate of 1/100,000 and this increased to 1/100 by 6-8 weeks post-infection[87]. Similarly, virus was detected in plasma around 3 weeks post-infection and continued to increase until approximately 6-8 weeks post-infection before rapidly falling to low or undetectable levels (typically associated with asymptomatic disease)[22,87,91]. At this stage, viral RNA is found in approximately 1/1000 peripheral blood mononuclear cells (PBMC) [87]. This rise and fall in viremia and PBMC infection coincided with the appearance and resolution of clinical signs of illness seen 6-8 weeks post-infection and with a decrease in CD4 T cells and expansion of $CD8\beta^{low}$ cells within lymph nodes and peripheral blood. This shift in viral RNA (free virus) to viral DNA (provirus) is indicative of the ability of the immune system to control but not clear infection[87].

Humoral immunity as measured by non-neutralizing or binding antibody production by activation of B cells to the p24 viral capsid protein was reported as early as 2 weeks following experimental infection[87]. This antibody response peaked about 6-8 weeks post-infection prior to the decrease in viremia and remained detectable life-long[87,92]. Production of FIV antibodies is aimed primarily at the V3 regions of the *env* gene due to its role in attachment of the virion to the cell but also the V4 and V5 regions[93]. As protection against the immune system, the *env* region is coated in N-glycosylation sites (carbohydrates that are linked to viral proteins) that mask the epitopes responsible for entry into the cell[93]. Non-neutralizing antibodies, which bind to viral epitopes exposed on the outer membrane of the virion, flag the antigens for destruction by immune cells but do not inhibit their infective capabilities. This is in contrast to neutralizing antibodies that

bind to viral epitopes and block infection by interfering with binding of the virion to its receptors, blocking virion uptake into a cell, preventing uncoating of the viral genome within a cell or causing aggregation of viral particles in circulation[84]. In FIV, similar to HIV, neutralizing antibodies develop slowly over time and have been reported to plateau at 50-60% neutralization ability at 4 years post-infection[93,94]. Broad neutralizing antibody activity, or the ability to neutralize multiple viral strains, is considered rare. In a recent study including 345 plasma samples from FIV-infected cats, detection of broadly neutralizing antibodies against a representative FIV subtype A (GL8) ranged from 0-100%; however, only 2 of 345 plasma samples from FIV-infected cats had broad neutralizing ability against a range of infectious subtype strains of FIV[93]. This suggests that humoral immunity does not play a strong role in combating the spread of infection to new cells.

2.5 Mechanisms of Immune Impairment

In uninfected healthy adult cats, CD4 and CD8 T cell counts have been reported as 1182 cells/μL (range: 543-1820) and 674 cells/μL (range: 353-994), respectively, with a CD4:CD8 ratio of 1.9 (range: 1.2-2.6) [95]. Following experimental infection, alterations within peripheral blood, lymphoid and non-lymphoid tissues are well characterized. Within the first 6-8 weeks of infection there is a marked decrease in CD4 T cell counts, commonly below the reference interval. CD4 T cell levels have been reported to be as low as 20% of pre-infection levels at the peak of clinical signs of illness[86]. Infected CD4 T cells are removed by direct cell-to-cell interaction with cytotoxic CD8 T cells. Apoptotic pathways (programmed cell death secondary to infection) are activated secondary to cellular infection and cell lysis secondary to rupture and release of new viral particles also serve to decrease CD4 T cell populations[84]. CD8 T cell counts also decline, but to a lesser extent [96]. CD4 T cell counts may recover during the asymptomatic phase, but not to pre-infection levels and in chronic infection this decrease in CD4 T cells typically does not become statistically significant until about 18 months after infection[20,97]. In one study of experimental infection, the mean CD4 T cell count was reported as 691±130 cells/mm^3 in FIV-infected cats compared to 1450±46 cells/mm^3 in FIV-uninfected cats at 18 months post-infection[98].

There is an overall inversion of the CD4:CD8 ratio throughout the remaining course of infection[20,22,23,99]. In one study, 2 experimentally infected cats were reported to have CD4:CD8 ratios of 0.62 and 0.49 at 103 weeks post-infection[23]. In a recent study of experimentally infected cats, the CD4:CD8 ratio inversion was reported to be statistically significant as soon as 6 weeks

post-infection and averaged 0.75±0.47 in chronically FIV-infected cats compared to 1.65±0.38 in FIV-uninfected cats[22]. Loss of CD4 T cells and decline of CD4:CD8 ratios are hallmarks of FIV infection. A subset of CD8 T cells ($CD8\beta^{low}$) increases in FIV-infected cats compared to uninfected cats[98,100]. The expansion of $CD8\beta^{low}$ cells begins within weeks of infection and they comprise up to 90% of the total CD8 T cell population at ≥7 years post-infection[100]. In a set of experimentally infected cats, $CD8\beta^{low}$ cells comprised 2.98 ± 0.8% (174±14 cells/mm) of peripheral blood mononuclear cells pre-infection compared to 17.31 ± 9.5% (1120±30 cells/mm) post-infection[98]. Absolute $CD8\beta^{low}$ cell count at 18 months post-infection has been reported to be 352 ± 60 cells/mm^3 in FIV-infected cats compared to 158 ± 60 cells/mm^3 in FIV-uninfected cats[98]. The $CD8\beta^{low}$ cells are considered activated effector cells as determined by the phenotype, $CD8\beta^{low}$CD62L-CD44+CD18+CD49+. This is similar to what occurs in HIV-infected people[101]. $CD8\beta^{low}$ cells suppress viral replication *in vitro* and are thought to be responsible for decreasing viremia in acute infection and maintaining low level viremia during asymptomatic infection in FIV-infected cats[102,103]. The exact mechanism of action is not known but the presence of $CD8\beta^{low}$ cells in culture with FIV-infected cells greatly reduced viral replication within cell culture supernatant[104]. Presumably this is done through cell-mediated immunity mechanisms that may include direct interaction with cytotoxic T lymphocytes (CD8 T cells) and production of cytokines (interferon-gamma and tumor necrosis factor) that protect cells against viral infection [105].

Cats that progress to the terminal stage of illness may exhibit marked immunosuppression and a lack of response to FIV viral replication and invasion by secondary pathogens[106]. During end stage disease a decline in FIV antibody production to undetectable levels, a resurgence in viremia, severe loss of CD4 T cells, and markedly decreased CD4:CD8 ratio have been reported compared to cats in the asymptomatic stage of infection[18,19,24,107] . In studies of naturally infected cats, viral loads were reported to increase 100-150 fold compared to cats in the asymptomatic stage of disease[24,107]. Significant declines in CD4 T cells (671±432 vs 147 ±103 cells/μL), CD8 T cells (492±314 vs 162±72 cells/μL) and CD4:CD8 ratio (1.45±0.53 vs 0.87±0.26) in cats in the asymptomatic stage of infection compared to cats in end stage infection have been reported[24]. In one study of experimental FIV infection, the most severe decline in reported for a cat in end stage disease was a CD4 T cell count of 21 cells/μL and a CD4:CD8 ratio of 0.075[108]. However, in another study of experimental infection, one cat in the chronic stage of infection had a CD4 T cell count of 42cells/μL and had no clinical signs of illness. This suggests that some cats may be able to tolerate low CD4 T cell counts without adverse side effects. Acute and chronic FIV infection

has been widely studied but the long duration of infection, particularly the lengthy asymptomatic stage of infection, makes longitudinal studies of infected cats reaching end stage disease difficult and expensive. Additionally, it is unknown what percentage of cats will progress to end stage disease and recent reports have indicated FIV-infected cats may likely succumb to other diseases before they succumb to FIV infection[14,67,68]. As such, the underlying factors that cause progression from asymptomatic to end stage disease remain elusive.

The underlying immune dysfunction in FIV-infected cats is reported to be the result of alterations in T cell populations, lymphoid tissue structure, cytokine production and immune responses to pathogens. However, the underlying cause of immune dysregulation and disease progression has yet to be defined. Several mechanisms have been suggested, including cytokine dysregulation, immunologic anergy, activation of immune regulatory cells and chronic immune activation[109].

Cytokines are secreted proteins that direct communication signals from one cell to another in order to direct an immune response. There are several classes of cytokines including: interleukins (communication signals between leukocytes), colony stimulating factors (support hematopoietic precursor proliferation), tumor necrosis factors (stimulates cytotoxicity) and interferons (interrupt viral replication)[105]. Cytokines are constitutively produced to help maintain immune homeostasis but are specifically up-regulated or down-regulated during an immune response. Kipar et al investigated the constitutive expression of several cytokines (interleukin-1β (IL-1β), IL-6, IL-10, IL12, and tumor necrosis factor-alpha (TNF-α)) from monocytes and macrophages in healthy cats over a range of ages (15 months to 10 years) and reported that all cytokines were constitutively expressed but this expression was variable within an individual cat and between cats of the same age. In general, the highest transcription levels were found in young adults and older cats for IL-1β, IL-6 and IL-12 whereas transcription levels of IL-10 and TNFα remained stable across different ages[110].

FIV infection alters cytokine production and response to various infections. Monocyte and macrophage constitutive cytokine transcription levels in experimentally asymptomatic FIV-infected cats were decreased compared to uninfected controls with only IL-1β found in all FIV-infected samples[111]. Alternatively, the PBMC transcription of these cytokines is markedly increased in symptomatic infection compared to asymptomatic infection[112-116]. In one study of FIV, the mRNA levels of interleukin 10 (IL-10) and interferon-gamma (IFNγ) from PBMC were highest at 6-8 weeks post-infection, with a transient peak in IL-4. This correlated with the

presence of clinical signs of acute infection. The mRNA cytokine levels then decreased, although not to the level of pre-infection, once the cats entered the asymptomatic phase. The CD4 T cells appeared to be the main source of IL-2, IL-4, IL-10, IL-12 transcription, and CD8 T cells were the main source of IFN-γ transcription[113]. In chronic FIV infection, both experimentally induced and naturally acquired, infected cats had increased plasma levels of IL-1β, IL-6 and TNF-α and had decreased IL-2 production and decreased PBMC proliferation in response to mitogen stimulation compared to uninfected cats[112]. The altered cytokine production and mitogen responses were more pronounced in symptomatic cats with clinical signs of illness (anorexia, weight loss, fever, stomatitis, lymphadenopathy, anemia, leukopenia and hyperglobulinemia) compared to asymptomatic FIV-infected cats[112]. When challenged with intracellular pathogens such as *Toxoplasma gondii* and *Listeria monocytogenes*, FIV-infected cats had less lymph node enlargement, broader pathogen dissemination, higher pathogen burden and decreased inflammatory cytokine production, all consistent with an increased susceptibility to infection and a decreased ability to clear infection compared to uninfected cats[113,114]. The alterations in cytokine production resulted in an increased IL-10:IL-12 ratio which may represent a cytokine shift in FIV-infected cats that favors a Th2 humoral immune response over a Th1 cell mediated response. This could contribute to impaired ability to quickly clear infection via cell-to-cell interaction and cytotoxic mechanisms[113,114,116]. A similar cytokine profile has been identified in HIV-infected people, including high pro-inflammatory cytokines INF-α, IL-1, IL-6, IL-18 and TNF-α, and is associated with innate immune cell activation, apoptosis of immune cells, and decreased lymphocytes levels that in turn decreases homeostatic cytokine production (IL-17, IL-22) from lymphocytes[117,118].

Initiation of an adaptive immune response is dependent on interaction with cells of the innate immune system. During this interaction an antigen presenting cell (APC), typically a dendritic cell or macrophage, bearing an epitope of the invading antigen binds to the T-cell receptor (TCR) specific for that epitope on a naïve T cell. Once bound to the T cell, a costimulatory signal between the APC and T cell is needed to direct the cell's immune response. In general, this costimulatory signal either propagates or dampens the inflammatory immune response. In every immune reaction both responses are needed – one to increase the immune response to clear the infection and the other to regulate the immune response to avoid unnecessary and prolonged tissue injury. In the pro-inflammatory response, B7.1 or B7.2 (CD80 and CD86, respectively) on the APC binds to CD28 on the T cell to initiate signal transduction and inflammatory cytokine production to direct humoral and/or cell-mediated immunity[84,105]. This co-stimulation causes

increased production and release of IL-2, up-regulation of IL-2 receptors, and activation of neighboring T cells to increase the response to the pathogen threat. In contrast, during the regulatory response, B7.1 or B7.2 on the APC binds to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on the T cell to induce a state of anergy (inactivity) or activate apoptosis. This reaction causes decreased IL-2 production and down-regulation of IL-2 receptor expression which eliminates the activation signal to neighboring T cells. Up-regulation of CTLA-4 on the T cell usually occurs 2 to 3 days after the initiation of the T cell immune response. Cellular anergy and apoptosis are important and necessary steps in regulating the immune response. Binding affinity of B7 to CTLA-4 has been reported to be 20-100X that of CD28 suggesting the importance of the regulatory response[84,105]. Several studies have reported that lymphocytes from lymph nodes and peripheral blood of FIV-infected cats have increased expression of B7.1, B7.2 and CTLA-4 and decreased expression of CD28 compared to those from uninfected cats[119-122]. These changes in expression patterns of CD4 and CD8 T cells have been reported to increase with age and time post-infection and are inversely correlated with lymph node CD4:CD8 ratio and peripheral CD4 and CD8 T cell counts[121]. Additionally, after 24 hours in culture, T cells from FIV-infected cats showed increased levels of spontaneous apoptosis compared to those of uninfected cats, particularly after stimulation with Concanavalin A[119-122]. The level of spontaneous apoptosis and anergy in stimulated cultured T cells can be decreased by either addition of exogenous IL-2 or antibody blockade of B7 and CTLA-4 receptors, particularly B7.1, although reduction of apoptosis is not complete[119,122]. A better response to apoptosis blockade has been reported in FIV-infected cats with viral RNA loads $<1x10^5$ compared to FIV-infected cats with higher viral RNA loads[119]. Based on the up-regulated expression of B7.1 and B7.2, it has been shown that activated T cells can act as APC and mediate apoptosis and anergy through T cell-T cell interactions between B7 and CTLA-4 binding[122].

Regulatory T cells ($T_{reg)}$ are a specific subset of CD4 T cells that carry the activated phenotype $CD4^+CD25^+CTLA4^+$ and are differentiated in the presence of TBGFβ and IL-6 using the transcription factor FoxP3[123-125]. These cells constitute about 5-10% of T cells in peripheral blood and 20-30% of T cells in lymph nodes in both FIV-infected and uninfected cats. T_{reg} cells have increased activity in FIV-infected cats[123], in both the acute and chronic states of infection, compared to FIV-uninfected cats and decrease the immune response to invading pathogens, however, the exact mechanism of increased activity is unknown[122]. It is known that once activated, these cells do not produce the cytokine IL-2 or up-regulate IL-2 receptors on the cell

surface and therefore fail to activate surrounding CD4 T cells to produce an appropriate immune response[123]. In culture, these cells have been reported to suppress the immune system by cell-cell interaction and down-regulate IL-2 production and receptor expression in neighboring T cells[123]. Chronic immune activation (immune hyper-activation) has been shown to occur in CD4 and CD8 T cell populations of FIV-infected cats. Phenotypic analysis of activation in the T cell populations of FIV-infected cats has revealed decreased CD8β chain immunofluorescence, down-regulation of CD62 L-selectin on CD4 and CD8 T cells, progressive loss of the CD8 naïve cell pool, and up-regulation of CD25[100,101,121]. These changes are consistent with the immunophenotype of a chronically activated immune system. Studies show that up to 70% of CD8 T cells and 80% of CD4 T cells have an activated phenotype in cats infected with FIV ≥7 years[98,100,101]. In addition to T cell hyper-activation, chronic B cell activation has also been reported in FIV-infected cats; this results in a polyclonal hypergammapathy secondary to increased antibody production[20,126]. The role that immune hyper-activation may play in disease progression has not yet been defined.

Together these changes in chronically FIV-infected cats – altered cytokine production, increased cell anergy and apoptosis, immunosuppression secondary to increased Treg activation and a state of immune hyper-activation – all contribute to an altered immune response and place the cats at increased risk for clinical disease.

2.6 Course of Infection

The course of FIV infection includes an acute phase followed by a long period of chronic infection during which progression from an asymptomatic to symptomatic state of infection is expected. In the acute phase of infection cats may experience transient signs of clinical illness. In experimental studies, lymphadenomegaly is reported as early as 10 days post-infection and may persist for months[22,69,86,112,127]. Further clinical signs of acute disease such as fever, diarrhea, malaise, anorexia and dehydration may occur approximately 6 weeks post-infection, with some cats requiring fluid support and antibiotic therapy[33,86,99,127]. These vague clinical signs may go unnoticed by the pet owner

As transient clinical signs resolve, cats enter an asymptomatic phase of infection. This asymptomatic phase can lasts months to years before progressing to a symptomatic phase of infection[22,69,97,127]. The symptomatic phase of infection is characterized by varied clinical signs including, but not limited to, diarrhea, stomatitis, periodontitis, gingivitis, upper respiratory infection, and other opportunistic infections[92,127,128]. During this period of symptomatic infection cats often respond to supportive therapy and antibiotic treatment and may transition between

asymptomatic and symptomatic states. Many of these cats eventually progress to end stage disease marked by weight loss, loss of body condition, generalized muscle wasting, lymphopenia, neutropenia, anemia, and / or bacterial, viral and fungal infections that are refractory to treatment or opportunistic[17,19,33,41,112,129,130]. Neoplasia, most commonly lymphoma, but also squamous cell carcinoma, myeloproliferative disease and fibrosarcoma are reported to occur at a higher incidence in FIV-infected cats[131]. Lymphoma has been the most commonly reported neoplasia in FIV infection and although a direct mechanism has not been identified it is suspected that viral interruption or transformation of the host cell cycle oncogenes or tumor suppressor genes is involved[17,92,131,132].

While the general disease course is similar among cats, course of disease and clinical signs seen differ between individual cats and between study groups. Age at the time of infection is thought to play a role in disease progression as neonatal kittens are reported to suffer more severe blood dyscrasias (neutropenia, leukopenia) and prolonged lymphadenopathy in the acute phase while aged cats appear to have delayed antibody response, lower T-lymphocyte levels and more severe chronic illness than young adult cats or kittens[133]. The underlying cause of the delayed immune response in aged cats is not known but may be related to or influenced by the immunosenescence of aging. Differences in dose, strain, route of infection, initial immune response to infection, and immune competency at the time of infection may also influence the progression of disease. In naturally acquired infection, the infectious dose, FIV strain, and initial immune response to infection are unknown which makes extrapolation of experimental data difficult. However, in a recent study of experimental FIV infection, it was reported that within groups of cats given the same infectious dose of one of three viral strains there was a variety of immune responses. The variation in immune response was dose-dependent and positively correlated with viremia and $CD8\beta^{low}CD62L^{neg}$ expansion over the first 12 weeks post-infection[134]. This supports previous findings that individual immune response to FIV infection was varied but appeared to be dose-dependent as cats receiving a lower infectious dose had a longer time to seroconversion and undetectable lymphadenopathy as compared to those that received higher infectious doses[135]. These results suggest that infectious dose and initial immune response may predict disease progression but long term follow-up in experimental settings has not been performed.

Experimental infection poses a unique situation in that FIV-infected cats are commonly housed under specific pathogen free (SPF) conditions which shield them from common pathogens that naturally FIV-infected cats may encounter. Similarly, housing conditions of naturally infected

cats may influence an individual cat's immune status and response to pathogen exposure. Crowded housing conditions and shelter housing have been associated with increased upper respiratory infection[136,137]. These housing conditions also carry increased risk of intestinal and dermatophyte pathogen exposure if proper hygiene protocols are not implemented and followed[138]. In a recent study, it was reported that FIV-infected cats living in a large multi-cat household had more clinical signs of illness on examination and shorter survival times compared to FIV-infected cats living in private homes of less than 2 cats, this suggests that management and housing play a role in FIV disease progression[139].

2.7 Diagnosis

Published guidelines recommend initial FIV testing by commercially available in-house ELISA tests be done for any cat that is: 1) new to a household, 2) showing signs of clinical illness, 3) living in a high-risk environment (outdoor cats, dynamic household or living with FIV-infected cats), or 4) FIV-uninfected and recovering from a cat fight. Retesting is recommended at least 60 days after an initial negative test in cats that are new to a household, have a recent history of fighting, after an initial positive test in kittens less than 6 months of age when maternal antibodies may still be circulating, or in cats with positive results and unknown history of FIV vaccination (which results in antibodies indistinguishable from those generated by natural infection). Annual testing is recommended for any FIV-uninfected cat living in a high-risk environment[46-48] .

Initial testing for FIV is typically performed using an in-house enzyme linked immunosorbent assay (ELISA) to detect antibody against FIV p24 surface protein. In the case of positive ELISA test results or high suspicion of disease in a cat with negative results, additional testing is recommended[46,47]. It is important to note that no diagnostic method for FIV is absolute therefore caution in interpretation of results is crucial.

False positive ELISA results can occur secondary to maternal antibodies in kittens less than 6 months of age, or in cats that have received an FIV vaccination. Available ELISA tests cannot differentiate between antibodies acquired via maternal transfer, antibodies secondary to vaccination, and antibodies secondary to active infection[140,141]. Cats which have received the FIV vaccine reportedly test antibody-positive on ELISAs for years following the last FIV vaccination[141-143]. In a recent study, it was FIV-uninfected cats that received the FIV vaccination tested antibody-positive on in-house ELISA tests as long as 11 years after the last FIV vaccination was given (Nichols et al, unpublished data). False negative ELISA results, although

less common, can occur early in infection prior to seroconversion, or in cats with end-stage FIV disease in which antibody production can be below a detectable level [144,145].

Polymerase chain reaction (PCR) tests available commercially differ by primer set and reaction protocol, and reported sensitivity and specificity vary[146,147]. PCR tests detect the presence of viral RNA and DNA and can detect acute infection prior to the development of an antibody response. False positive PCR results can occur, for example, secondary to contamination at the time of sample collection or during processing at the laboratory. False negative PCR results can occur, for example, in cats with viral loads below that which can be detected by PCR. Detection limit is defined as the lowest number of viral copies that can be identified in a standard sample; detection limits vary between laboratories but can be as low as 1 to 10 copies in a given sample. Additionally, as primer sets and protocols differ between laboratories it is possible that a specific primer set will not detect all FIV strains. One PCR comparison study, between 3 different laboratories, reported sensitivities between 41 and 93% and specificities between 81 and 100% in correctly identifying cats infected with FIV subtypes A, B and C from those not infected[147]. Another study, using 3 different laboratories in Canada, found similar varied results[146]. Potential reasons for variation in reported sensitivities and specificities included sample contamination during sample processing, differing primer sets, detection limits and sample type (whole blood, plasma, serum or blood smear)[143,146,147].

Western blot serologic testing has been used as a confirmatory test for FIV infection. In this test, FIV core viral proteins, namely p24 and p15, are separated by size using gel electrophoresis and transferred to a membrane. The membrane is then incubated with a serum sample and a secondary fluorescent reagent to detect binding between viral proteins and sample antibodies. Binding of at least 2 separate viral proteins is needed to report a sample as positive; binding of only 1 protein is considered indeterminate[143]. Reported sensitivity and specificity are typically lower than those of ELISA tests[144,146]. False negatives can occur due to viral protein multimeric binding which creates viral proteins of larger size that will not be correctly identified as FIV viral core proteins[143]. Similar to other serologic testing, if the test is performed prior to an adequate antibody response a negative result may occur[143].

Virus isolation (VI) is considered the reference standard. Peripheral blood mononuclear cells (PBMC) isolated from FIV-infected cats are cultured with primary lymphoblast cells from SPF uninfected cats to determine whether infection is propagated from the PBMC cells to the blast

cells. Cultures are monitored for cell viability, cytopathic effect (ballooning degeneration, syncytium formation, and increased cell death), and Mg^{2+}-dependent reverse transcriptase activity in the culture supernatant. A reaction is considered positive when there is a five-fold increase of reverse transcriptase activity in the culture supernatant of 2 consecutive samples as compared to negative control culture supernatant. Identification of the virus in supernatant by electron microscopy, antigen ELISA or IFA can be done[148,149].

Virus isolation can be falsely negative as the test is technically challenging to perform and the outcome of the test is largely based on viral activity over time making the duration of culturing time crucial. Similar to PCR, false positive results may occur secondary to sample contamination. False negative results may be due to several factors that influence duration of the procedure including: 1) circulating virus numbers, 2) the reverse transcription (RT) activity within a culture, 3) viability of the virus and cells sampled from the infected cat, and 4) proper condition and maintenance of the culture cells and supernatant. Cats with high viral load would be expected to have a positive virus culture result sooner than cats with low viral load. The level of reverse transcriptase activity can also influence the speed at which VI may yield a positive result as increased RT activity would be identified sooner. Therefore, it is recommended that cultures be grown for 5 to 6 weeks before they are declared negative[148].

2.8 Hematology and Histopathology Alterations Secondary to Infection

2.8.1 Hematology

Hematological and serum biochemical alterations have been noted in FIV-infected cats. These alterations can fluctuate over time depending on other factors such as concurrent illness, environmental stressors, and age. Information regarding hematologic alterations in acute infection has largely been gleaned from experimental infection and in general, cats have leukopenia, neutropenia and sometimes lymphopenia as these cells respond to initial infection. Neutropenia is most likely to occur during acute infection as neutrophils are lost from circulation through diapedesis into tissues to respond to FIV-related inflammation[20,22,33,150]. Lymphopenia is the result of apoptosis and destruction of infected CD4 T lymphocytes following infection. In either natural or experimental infection, during the asymptomatic phase, FIV-infected cats may have lower mean leukocyte counts compared to FIV-uninfected cats but FIV-infected cats do not generally have a true leukopenia; cell counts remain within the reference interval but at the lower limit[20,22]. During the symptomatic phase, particularly end stage disease, marked anemia, leukopenia and thrombocytopenia may occur[18,19,150]. In either setting, serum biochemistry abnormalities most

commonly include hyperproteinemia attributed to a polyclonal hypergammaglobulinemia, thought to occur secondary to B cell stimulation and antibody production to varied FIV epitopes [20,68,69]. Mild hypernatremia has also been reported but the underlying cause remains unknown[68]. Decreased urine specific gravity (USG) has been reported in naturally and experimentally infected cats without concurrent azotemia[151]. Additionally, naturally infected cats were reported to have a higher urine-to-creatinine (UPC) ratio compared to client-owned uninfected cats whereas experimentally infected cats had an increased creatinine level compared to SPF housed cats[151]. The reason for the difference between naturally and experimentally infected cats is unknown.

2.8.2 Histopathology

Histologic changes within lymph nodes, thymus, spleen, bone marrow, gut-associated lymphoid tissue (GALT), intestinal epithelium, kidneys, and brain have been reported. Alteration and compromise of structural integrity of these organs, particularly lymphoid organs, are seen as early as 3 weeks post-infection[86,87].

In experimental infection, the most severe lesions appear in lymphoid tissues at approximately 6-8 weeks post-infection and coordinate with the appearance of clinical signs[86]. Changes are most dramatic in thymus, bone marrow, lymph nodes, spleen and mucosal associated lymphoid tissue (MALT) [86,87]. Grossly, lymph nodes are enlarged and the thymus may be smaller in size compared to similar tissues in uninfected cats. Histologically, the thymus shows cortical involution, decreased thymocyte numbers and thymitis characterized by infiltration of lymphocytes and macrophages into the interlobular area[86,87]. The bone marrow is hyperplastic with increased cellularity characterized by increased granulocyte and monocyte precursors although the myeloid:erythroid ratio remains normal[86,87 150]. In peripheral lymphoid tissues, a progressive hyperplastic change is characterized by increased number and size of lymphoid follicles, prominent germinal centers, prominent high endothelial vessels (HEV) containing lymphocytes and expansion of parafollicular domains that contain lymphocytes, macrophages and lymphoblasts[86,87]. Similar findings are reported in adult cats naturally infected with FIV[28]. Histologically, lymph nodes show follicular hyperplasia but also follicular involution (cortical sinus collapse, small to non-existent germinal centers and eosinophilic hyaline deposits) or a mixture of both[28]. These central and peripheral lymphoid tissue histological findings are similar to those reported in people with chronic HIV infection.

Changes occur in other tissues. The histologic lesion most commonly reported is a perivascular inflammatory infiltrate, comprised of lymphocytes, neutrophils, plasma cells and macrophages in the small and large intestines, oral cavity, kidney, liver, lung, eyes and brain. Small intestinal villous blunting, loss of villi, crypt dilatation, and necrotizing enteritis is reported[28]. Additional secondary pathogens within the intestinal tissue that may be, in part, responsible for these lesions have not been identified[28]. Amyloid deposition in the organs of cats naturally infected with FIV has been reported in some naturally infected cats, but this is not reported in experimentally infected cats do not. A specific cause for this discrepancy is not known[152,153]. Renal amyloidosis is reported in HIV infection and, although considered uncommon, may be a contributing factor to the increased incidence of renal disease in HIV-infected patients[154].

2.9 Monitoring and Therapy

Current recommendations for monitoring of cats infected with FIV include twice yearly physical examination and once yearly routine hematological and serum biochemistry panels and urinalysis[46,47]. These monitoring techniques identify illness and allow medical intervention only after disease has occurred. Currently, there are no readily available and reliable techniques to monitor FIV-infected cats to indicate progression of disease and when medical intervention should be initiated to prevent development of clinical disease.

This is in stark contrast to the monitoring techniques and treatments available for HIV-infected people, for whom infection and disease progression has been traditionally monitored by measurement of CD4 T cell counts, viral loads, and evaluation of clinical signs. This information is used to decide when medical intervention is necessary and to gauge response to treatment[34,35]. The mainstay of HIV therapy is a combination of various anti-retroviral medications, known as highly active anti-retroviral therapy (HAART), tailored to the individual patient[155]. HAART therapy results in decreased viremia and increased CD4 T cell counts. The medications work by interrupting viral replication, at different stages, which reduces viral particle burden in tissues and peripheral blood. There are several points during viral replication at which antiretroviral therapy can interfere with replication including: 1) attachment of the virus to the host cell surface receptors, 2) fusion of the viral particle with the host cell, 3) reverse transcription of viral RNA, 4) nuclear translocation and viral dsDNA integration, 4) nuclear export of viral mRNA, and 5) viral assembly and maturation of the virion in the cytoplasm. In HAART, the most commonly used class of drug is the nucleoside reverse transcription inhibitors (NRTIs). The general mechanism of these compounds is to generate altered deoxynucleotides (dNTs) that compete with

cellular dNTs for insertion into the elongating viral DNA strand. Once incorporated, these altered dNTs are unable to bind the next dNT in sequence and effectively halt production of that viral DNA strand. The first of these compounds to be approved for use in anti-retroviral therapy was azidothymidine (AZT) or Zidovudine. Since then, many other NRTI class drugs have been cleared for use in HIV-infected patients that each have slightly different mechanisms of action, reduced patient side effects such as anemia, and better tolerance of viral mutations[50].

Positive response to HAART therapy results in decreases provirus burden and increases CD4 T cells numbers in lymphoid tissues and peripheral blood of HIV-infected people. The decreased viral load lessens the burden of free virus that can infect new CD4 T cells which results in fewer CD4 T cells being removed by cytotoxic CD8 T cells and apoptosis, allows for restoration of the CD4 T cell count, and decreases the number of CD4 T cells acting as a reservoir of viral infection. The restoration of CD4 T cells, even though it may be incomplete, strengthens the ability of the adaptive immune system to respond to routine pathogen encounters in the mucosal tissues and within the lymph nodes. In most patients showing a positive response to HAART, there has been a significant increase in survival time and quality of life and a decrease in clinical signs of illness[155] . There are some patients, though, that show progressive disease despite a positive response to HAART. In these patients, progressive disease and shorter survival times has been associated with increased immune activation rather than CD4 T cell counts or viral burden[38].

In FIV-infected cats several antiretroviral therapies have been investigated. Although two drugs have recommended doses for therapy, they have limited scope in use[47]. Zidovudine (AZT), the NRTI described above, is fairly well tolerated in cats and is effective in reducing clinical signs, decreasing viral burden and stabilizing CD4 T cell counts. However, in some cats it produces a non-regenerative anemia. Resistance to the drug is common and can occur as soon as 6 months after initiation. Drug resistance is secondary to the poor proof-reading and editing capabilities of FIV reverse transcriptase which allows for mutation of the viral genome and loss of the drug's site of action. Another drug, AMD 3100 or Plerixafor, is a CXCR4 antagonist and blocks binding of the viral particle to the CXCR4 receptor. This prohibits fusion and entry of the virus particle into the host cell. The drug appears to be well tolerated in cats, results in a decrease in proviral load compared to a placebo, and does not show evidence of drug resistance. However, there were no reported significant decreases in clinical signs of disease in one study[44]. Other drugs that have been investigated, PMEA and D4API, have caused severe anemia or liver disease which in some cases was fatal[45].

2.10 Microbial Translocation

Microbial translocation is defined as the nonphysiological passage of gastrointestinal (GI) microflora through the intestinal epithelial barrier and lamina propria, with eventual dissemination to the mesenteric lymph nodes and then extranodal, sites[40]. Microbial translocation is believed to have an important role in chronic immune activation of HIV-infected persons. When it became apparent that some HIV-infected persons still succumbed to progressive disease despite a positive, appropriate response to HAART, investigation showed that in some HIV-infected persons, shorter survival times were not linked to increased viral burden or decreased CD4 T cell counts. Rather, these individuals had high levels of immune activation, as measured by markers of lymphocyte activation, and this immune activation better predicted progression of disease and a shorter survival time[38]. It was postulated that chronic immune activation led to immune exhaustion which in turn led to increased susceptibility to pathogens, enhanced other disease processes, and ultimately led to disease progression[26,39].

Brenchley et al.[26,39] proposed that microbial translocation from the gastrointestinal tract into circulation as a source of chronic immune activation and disease progression as opposed to immune activation by HIV alone. In health, the gut associated lymphoid tissue (GALT) of the gastrointestinal tract (GIT) contains approximately 80% of the lymphoid tissue in the body, likely due to the constant exposure to microbial products at the interface of intestinal epithelial cell and luminal contents. Within the GIT, several mechanisms create immune barriers to further protect the body against luminal microbes including: epithelial tight junctions between cells prevent microbial movement between cells, a mucus layer produced by goblet cells prevents microbes from adhering to epithelial cells, secretory Immunoglobulin A (IgA) identifies pathogens within the intestinal lumen for removal, and an immune cell dense lamina propria directly beneath the intestinal epithelium layer[27,156].

In a healthy GIT, most microbial products that cross the epithelial barrier are contained and removed by macrophage phagocytosis within the lamina propria and mesenteric lymph nodes[26,40]. In pathogenic lentiviral infections, the integrity and structure of the GIT and GALT is compromised, enabling microbial translocation[26,40]. In non-human primates infected with pathogenic SIV, structural loss of tight junctions between intestinal epithelial cells, small foci of epithelial cell erosion, increased crypt enterocyte turnover and evidence of lymphocytic-plasmacytic inflammatory infiltrates occur 14-28 days post-infection and continue into the chronic stage of disease[157]. In HIV and SIV-infected individuals, GALT undergoes severe CD4 T

cell depletion during the acute phase of infection that exceeds the level of CD4 T cell depletion seen in peripheral blood and lymph nodes[25,158,159]. Furthermore, restoration of CD4 T cell counts of GALT is delayed and incomplete even at 5 years post-infection[25,158]. The CD4 T cell population of GALT contains a larger proportion of CCR5+CD4+ T cells, the primary target for HIV and SIV, than other lymphoid tissues and peripheral blood and likely serves as a reservoir for infection and viral replication. The CD4 and CD8 T cells within GALT of HIV-infected patients, also express a higher proportion of activated phenotypes (CD25+, CD27-) at the expense of naïve or resting T cell phenotypes contributing to the risk for microbial translocation, compared to HIV-negative patients[25,158,159].

SIV-infection of non-human primates is pathogenic in certain species and not in others. In either case, infection causes marked deletion of T cells in intestinal mucosal and peripheral lymphoid tissues as well as lymphoid hyperplasia and lymphoid fibrosis[160,161]. However in species for which the virus is not pathogenic, infected animals do not have the same degree of intestinal barrier compromise or deleterious secondary infections as seen in animals for which the virus is pathogenic. In a study by Estes et al[157], it was shown that during pathogenic SIV infection, Gram-negative bacterial products, including lipopolysaccharide (LPS), were present in the lamina propria of GALT adjacent to the sites of epithelial barrier compromise. Gram-negative bacterial products were also identified in mesenteric lymph nodes draining the gastrointestinal tract (GIT), axillary lymph nodes and liver. LPS was identified in the medullary cords, sinuses, paracortex and germinal centers of affected lymph nodes. There was a positive correlation between the quantitative amount of LPS within the lamina propria and mesenteric lymph nodes and axillary lymph nodes. There were no bacterial products in the lamina propria of GALT, GI lymph nodes, peripheral lymph nodes or liver in the non-human primates for which the virus is not pathogenic. This supports the theory that alterations in lymphoid tissue T cell populations and structural compromise of the intestinal epithelial barrier in pathogenic infection permit primary bacterial translocation. Once disseminated, these bacteria and bacterial products are a source of chronic systemic immune activation.

The innate immune system functions as the surveillance system to identify pathogens. The macrophages, neutrophils and dendritic cells utilize pathogen recognition receptors (PRR) on their outer membrane to identify highly conserved epitopes, termed pathogen associated molecular patterns (PAMP), located on pathogens. These receptors include toll-like receptors (TLR) that recognize certain bacteria, viruses and fungi, and glucan, mannose, and scavenger

receptors that recognize certain cell wall components of bacteria, yeast and fungi. The presence of PAMPs, found only on the outer membrane of pathogens, serve as a danger signal to alert the immune system to a foreign invader. Examples of PAMPs include lipopolysaccharide (LPS) of Gram-negative bacteria, lipotechoic acid (LTA) of Gram-positive bacteria and mannose-rich oligosaccharides of yeast and fungi. Once a PRR has bound to a PAMP it starts a cascade of events including pro-inflammatory cytokine production, extravasation of innate cells into tissues, chemotaxis of cells to the site of infection and antigen presentation to cells of the adaptive immune system[84,105].

Gram-negative bacteria are a primary component of the microbial population within the GIT. LPS, a PAMP on Gram-negative bacteria, is a potent immunostimulatory agent and is readily recognized by PRRs on marcophages of the innate immune system, in particular TLR-4 and CD14. The immune signaling pathway caused by the presence of LPS is complicated and not fully understood, however, it is known that multiple protein molecules play a role in the signaling cascade. Currently, it is known that the lipid A tail of LPS is the potent immunostimulatory region of the antigen. The lipid A portion is bound to the N-terminus of lipopolysaccharide binding protein (LBP), an acute phase serum protein that is produced by hepatocytes, epithelial cells and muscle cells. It is the C-terminus end of LBP that binds to CD14 to complete the trimerization of LPS-LBP-CD14. Once this trimer is formed, further signaling through the TLR-4/MD-2 complex on the macrophage is carried out to produce pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, and tumor necrosis factor alpha (TNF-α)[162]. In low concentrations of LPS, LBP extracts LPS from the Gram-negative membrane and transfers it to CD14. In high concentrations of LPS, LBP will shuttle LPS to serum lipoproteins or form LPS aggregates for removal from the body through other mechanisms[162-167].

CD14, a key glycoprotein in the immune signaling cascade following LPS detection, is expressed by monocytes, macrophages and neutrophils and occurs in membrane bound (mCD14) and soluble forms (sCD14)[162,163]. Although sCD14 is most notably associated with LPS activation there is also evidence that it can enhance LTA, lipoarabinomannanes, peptidoglycans and other glycoprotein signaling[168]. Soluble CD14 is necessary for an immune signaling response in low concentrations of LPS. In addition to the trimerization pathway outlined above there is a second pathway that sCD14 is thought to employ to initiate an LPS signaling cascade. In this second pathway, sCD14 binds LPS in circulation and shuttles it to the MD-2 receptor of the macrophage after which sCD14 is released and recycled back into circulation. The LPS bound to MD-2 causes

a conformational change in the TLR-4 receptor on the surface of the macrophage which in turn carries out the LPS intracellular signaling cascade and pro-inflammatory cytokine production. It is thought that at high LPS concentrations sCD14 may act similar to LBP and shuttle LPS to lipoproteins for removal from the body[163].

Recently, LPS, sCD14 and LBP have been identified as plasma biomarkers of immune activation that can be used to monitor for progression of disease in HIV-infected patients[26,40,169]. Plasma concentrations of sCD14, LBP and LPS are increased in HIV and SIV infected individuals compared to uninfected individuals and are further increased in chronically infected individuals compared to acutely infected individuals[26,169-172]. Soluble CD14 in plasma has also been shown to be an independent predictor of mortality in HIV-infected patients and is positively correlated with T cell activation in early HIV infection[173].

Bacterial 16S rRNA products have been found in peripheral blood samples of chronically HIV-infected patients with high plasma concentrations of sCD14, LPS and LBP, further indicating microbial translocation is an underlying cause of immune activation and disease progression [26,37,174-179].

Measurement of circulating LPS and bacterial 16S rRNA are considered direct evidence of microbial translocation, but use of these tests can be challenging[40,171]. Samples can easily become contaminated with bacteria or LPS at collection or during handling. Detection of bacterial 16S rRNA is also dependent upon primer choice, PCR reaction conditions, and amount of microbial products in circulation. The assays for LPS are technically challenging to perform and results are often difficult to replicate. Soluble CD14 and LBP are considered surrogate markers for microbial translocation as they are produced secondary to immune stimulation by LPS, and are positively correlated with plasma and serum LPS concentration[40,171]. Plasma LBP and sCD14 are measured using enzyme-linked immunosorbent assays (ELISA). Results of these assays have been replicated within and between laboratories making them more robust markers of microbial translocation than LPS quantitation or 16S rRNA PCR assay[40].

Chronic immune activation as an underlying cause of disease progression in HIV-infected patients is now generally accepted. Microbial translocation as an underlying cause of chronic immune activation is now recognized as a significant factor in disease progression for HIV-infected patients. Although the traditional monitoring techniques, CD4 T cell counts and viral load levels, are still the cornerstones of HIV patient care, the use of plasma biomarkers of chronic

immune activation as a monitoring tool is increasing and some researchers and clinicians suggest that it will become a routine part of HIV patient care.

Given the similarities between FIV and HIV - depletion of T cells, alteration in cytokine profiles, and chronic immune activation - exploration and investigation into microbial translocation in FIV-infected cats is prudent. Research into a feline specific immunoassay to assess the level of circulating sCD14 in FIV-infected cats is ongoing and preliminary results show promise for use of this assay in detecting increased inflammation associated with FIV disease progression (Dr. Gregg Dean, personal communication). Discovery of an easy and readily available biomarker of immune health, in contrast to T cell levels and viral loads, would potentially allow for better characterization of immune health and disease stage, improved and focused medical interventions prior to development of clinical signs of disease, and enhanced length and quality of life. This would not only impact the lives of client-owned FIV-infected cats but also those infected cats residing within shelters. Improved ability to determine and monitor the immune health of FIV-infected cats within shelters would provide beneficial information that could improve the adoptability, care and management of these cats.

2.10 Future Treatment

As microbial translocation has become an accepted pathway for chronic immune activation in HIV infection, the composition and relative abundance of bacterial species within the intestinal microbiome has become more relevant. With the invention of high-throughput sequencing, investigation into the intestinal microbiome has become increasingly easier and more common. Dysbiosis, fluctuations in bacterial population and composition, of the intestinal microbiome has become a focus of pathology in several diseases associated with a pro-inflammatory state including irritable bowel syndrome, diabetes mellitus and HIV.

In recent years, several studies have examined the microbiome of HIV-infected patients compared to uninfected controls and revealed significant differences between the two groups[180-182]. In general, researchers have reported an increase in *Pseudomonas aeruginosa* and *Candida albicans* and a decrease in Lactobacillus and Bacteroides spp. in fecal samples of HIV-infected individuals compared to uninfected controls[180-182]. Despite the difference in abundance of specific bacteria both groups had an equal richness in microbial diversity[180,181]. Differences in bacterial abundance based on sampling techniques such as mucosal biopsy, rectal swab or fecal sample are of concern. A recent study compared the microbiome of colonic mucosal samples to rectal swabs and fecal samples within individual patients, both HIV-infected and uninfected controls, and

reported that bacterial populations determined by sequencing techniques were not the same between sampled groups or sample types[180]. Colonic mucosal samples contained a higher abundance of Prevotella spp in the HIV-infected patients compared to uninfected controls. Prevotella spp were of much lower abundance in swabs and fecal samples in HIV-infected patients compared to colonic mucosal samples[180]. Prevotella spp are a Gram-negative pathobiont bacteria associated with other GI inflammatory diseases and are known for their ability to degrade mucin at the epithelial lining, disrupt epithelial tight junctions and contribute to intestinal barrier dysfunction[180]. Whereas the alterations in Bacteroides and Lactobacillus spp in rectal swabs and fecal samples were consistent between sampled group and sample type and also consistent with findings reported in similar studies[181,182].

In addition to bacterial composition and abundance, several studies have reported associations between certain bacterial species and elevations in markers of immune activation. Certain bacterial species, including Prevotella and Enterobacteria spp, have been associated with increased immune parameters including plasma viral load, colonic viral load, and plasma IL-6, IL-1β, LPS, and sCD14 concentrations[180,181]. But further studies must be done to establish a cause and effect relationship between dysbiosis and immune activation.

The acceptance of microbial translocation as an underlying cause of immune activation has ushered in an additional approach to HIV therapy aimed at supporting immune health and integrity. These approaches aim to decrease HIV pathologic sequelae including: viral replication within the GI mucosa, microbial translocation, lymphoid fibrosis and immune activation[183,184].

The use of raltegravir burst therapy in combination with traditional HAART medications has been shown to decrease reservoir viral replication within the GI mucosa and support restoration of CD4 T cell populations and decrease local inflammation[183]. Several studies have reported beneficial effects of prebiotics and probiotics in supporting the abundance of “good bacteria” such as Lactobacillus spp within the GIT resulting in increased GI mucosal CD4 T cell populations, deceased GALT lymphoid fibrosis and decreased T cell turnover within GALT[185,186]. One study in HIV-infected children, diagnosed with failure to thrive despite HAART medications, reported an increase in height and weight gains after 5 months of probiotic therapy compared to a placebo group[187].

The use of LPS binding agents in SIV-infected patients, such as sevelamer, have been reported to block microbial translocation by clearing LPS from the GIT and stabilizing sCD14 and LPS

concentrations as compared to those patients not receiving the drug in which sCD14 and LPS levels continued to increase[188]. Further studies investigating the use of medications to influence bacterial colonization and inflammation within the gut of HIV-infected patients are ongoing and include: the antibiotic, rifaximin, which concentrates in the GIT; the anti-inflammatory, mesalamine, that concentrates activity in the GIT; and chloroquine that inhibits toll-like receptor immune activation[183,184,189]. Additionally, the angiotensin converting enzyme inhibitor, Lisinopril, is being studied for its potential role in reducing lymphoid fibrosis by reducing angiotensin II which is reported to be associated with fibrosis by increasing levels of TGF-β1[183].

To date, characterization of the intestinal microbiome in FIV-infected cats is limited and investigation into therapies aimed at modifying immune activation in FIV-infected cats are lacking. One study reported no difference in microbiome richness, diversity or evenness between FIV-infected cats and uninfected cats; but FIV-infected cats had a higher percentage of Bifidobacteriales, Lactobacillales and Aeromonadales at the order level[190]. Based on operational taxonomic units, there was diversity in community membership and structure in FIV-infected cats compared to uninfected cats[190]. The difference in bacteria type compared to bacterial function was not performed. Investigation into the use of probiotics in FIV-infected cats showed decreased bacterial translocation in an ex vivo evaluation of small intestinal mucosa, suggesting some probiotics may be effective supportive therapy[191]. Several studies investigating the use of recombinant feline interferon-omega have reported clinical improvement and decreased viral shedding of upper respiratory pathogens in FIV-infected cats but none have reported an improvement in immune status such as CD4 T cell counts and viral load[192-194].

2.11 Conclusion

Feline immunodeficiency virus is an important, widespread, global disease in cats that causes progressive immune compromise which results in increased susceptibility to opportunistic infection, neoplasia and early death. Similar to human immunodeficiency virus (HIV) in people, FIV-infected cats have alterations in T cell counts, cytokine alterations, chronic immune activation and a decreased response to pathogens. Alterations in T cell populations, cytokine production and immune activation in the acute and asymptomatic stages of experimental FIV infection have been well documented and further understanding of the mechanisms associated with these changes is ongoing. However, despite these great strides in understanding this disease, the factors that propel a patient from asymptomatic to end stage disease are still unknown.

Unlike HIV-infected people, FIV-infected cats do not have access to advanced routine monitoring techniques, like CD4 T cell counts and viral loads, to identify progression of disease. This puts FIV infected cats and the veterinarians who care for them at a disadvantage. Investigation into inflammatory and lymphocyte immunophenotype health markers currently available is needed. Development of health panels that specifically monitor health parameters associated with inflammation such as white blood count, neutrophil count, albumin and globulin levels and CD4 T cell and CD8 T cell counts should be pursued. Additionally, optimizing the frequency for which FIV-infected cats should be examined and monitored should also be performed. This would allow veterinarians to identify early alterations in inflammation and immune cell activity and intervene with medical therapies or preventative treatment earlier to extend good quality of life.

Recent work in the study of HIV has found that chronic immune activation is associated with disease progression and early mortality. As such, new techniques to determine factors associated with immune activation have been developed for use in HIV-infected people. These techniques have the potential to advance patient monitoring, identify disease progression earlier, and guide future medical intervention to prolong health, thwart disease progression and increase quality of life.

Given the similarities between FIV and HIV, it is plausible that these same techniques will be applicable to FIV-infected cats and will provide an easy diagnostic technique to identify disease progression. Continued investigation into the underlying mechanisms of disease progression will lead to novel therapies aimed at reducing immune activation and improved management protocols for FIV-infected cats that will increase quality and quantity of life.

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CHAPTER 3. TEMPORAL PATTERNS IN GENERAL HEALTH PANEL MARKERS AND LYMPHOCYTE IMMUNOPHENOTYPE IN CATS NATURALLY INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS

3.1 Introduction

Feline immunodeficiency virus (FIV) is a complex, important lentiviral infection of cats. FIV infection causes immunosuppression and may lead to an increased risk of certain opportunistic infections, neoplasias and early mortality. The overall prevalence of FIV is reported to be approximately 2.5% in the United States [1,2]. Transmission of FIV among cats occurs primarily via inoculation of virus via penetrating wounds. Male, intact, outdoor cats are at increased risk for acquiring FIV infection due to territorial fighting behavior[3]. Transplacental and transmammary transmission in experimental settings has been reported but this is not considered a common mode of transmission in naturally acquired infection.[4,5]

FIV infections typically progress through three stages: acute, chronic (asymptomatic) and symptomatic (end stage). During the acute phase of infection cats may have anorexia, diarrhea, lethargy, fever and lymphadenomegaly beginning approximately 6 weeks post-infection[6,7]. The acute phase may last for several weeks and is followed by the asymptomatic phase during which cats show no outward signs of infection. The asymptomatic phase typically lasts for a variable period of years. During chronic infection, there are changes in the immune system of infected cats, and cats may be at risk for illnesses such as gingivostomatitis, upper respiratory infections and diarrhea, although these typically respond to treatment. Some cats progress to a final symptomatic stage of infection which is marked by an ongoing loss of body condition, treatment-refractory opportunistic infections, blood dyscrasias, neoplasia and death.[3,8]

During the acute phase of infection, cats may be anemic, leukopenic, lymphopenic and / or neutropenic but these abnormalities are transient. Cats in the chronic phase of infection may have relative leukopenia, lymphopenia and / or monocytopenia compared to FIV-uninfected cats but

values for asymptomatic FIV-infected cats typically remain within the reference interval[9,10]Additionally, hypergammaglobulinemia believed secondary to polyclonal B cell proliferation and increased immunoglobulin production has been reported in FIV-infected cats compared to FIV-uninfected cats[9,11,12]. Hypernatremia was reported in some FIV-infected cats although the cause for this was unknown[11]. For those cats progressing to end stage disease, marked anemia, leukopenia, neutropenia and lymphopenia are most commonly reported although reports of blood dyscrasias in symptomatic infection are sparse[13,14].

It is well documented in experimental infection that CD4 T cell percentages and absolute cell counts may markedly decrease during the acute phase of infection, rebound during the early asymptomatic phase (typically not to pre-infection levels) and then gradually decline over the remainder of infection[10,15-18]. The CD8 T cell population has been shown to increase following infection and this is reportedly due to an expansion of the CD8 T cell subset, $CD8\beta^{low}$ [17,19]. $CD8\beta^{low}$ cells are thought to be activated effector cells as based on phenotype and have been shown to decrease FIV replication *in vitro*; these cells may increase as early as 3 weeks post infection and are reported to comprise up to 70% of the total CD8 T cell population[19,20]. In one experimental infection, CD4 T cell counts of FIV-infected cats 18 months post-infection were 691±130 cells/mm^3 compared to 1450±46 cells/mm^3 in FIV-uninfected cats[17]. In this same population of FIV-infected cats, $CD8\beta^{low}$ cells were reported as 352±59 cells/mm^3 at 18 months post-infection compared to 158±61 cells/mm^3 in FIV-uninfected cats[17]. The decrease in CD4 T cells and increase in CD8/$CD8\beta^{low}$ cells, results in an inversion of the CD4:CD8 ratio in FIV-infected that persists throughout infection[9,10,18,21]. In one study of experimentally infected cats, the CD4:CD8 ratio inversion was reported to be statistically significant as early as 6 weeks post-infection and at each time point thereafter compared to uninfected control cats. The averaged CD4:CD8 ratio was reported as 0.75±0.47 in chronically FIV-infected cats compared to 1.65±0.38 in FIV-uninfected cats[10].

The majority of information detailing progressive hematological, biochemical and lymphocyte immunophenotype abnormalities of FIV-infected cats has been gained through studies of experimental infection. While experimental infections are very important to allow controlled study of the course of FIV infections, it is not known whether experimental infection accurately mimics natural infection. Experimentally infected cats are often inoculated as kittens, whereas it is believed that the majority of naturally infected cats are infected with FIV as adult cats. Experimentally infected cats are housed in specific pathogen free (SPF) conditions, and often

inoculated via intraperitoneal, intravenous or mucosal routes rather than deep, penetrating bite wounds. SPF housing conditions limit environmental pathogen exposure and protect cats from exposure to common pathogens encountered by naturally infected cats. It is not known whether experimental FIV inoculation doses and strains are similar to those encountered in natural infection. It is therefore difficult to make generalizations or predictions about disease progression in naturally acquired infection based on results from experimental infection.

The purpose of this study was to compare the temporal patterns of common hematological, plasma biochemical and lymphocyte immunophenotype values between FIV-infected cats and FIV-uninfected cats and also between FIV-infected cats residing in private homes and infected cats residing in sanctuary housing over a period of up to 4 years . The study was designed to test the following hypotheses: in comparison to FIV-uninfected cats, FIV-infected cats will have different temporal patterns in: 1) CD4 and CD8 lymphocyte numbers and ratios; 2) hematological parameters and 3) plasma biochemical parameters. Furthermore, there will be different temporal patterns in these parameters in FIV-infected cats residing in sanctuary housing compared to FIV-infected cats residing in private homes of <7 cats.

3.2 Materials and Methods

3.2.1 Animals

Cats were enrolled from adoption guarantee shelters (PAWS Chicago and Treehouse Humane Society, (Chicago, IL)), the Fitzhugh B. Crews FIV Sanctuary (Memphis, TN), or private homes in the Chicago, IL and Memphis, TN metropolitan areas. Cats were considered FIV-infected when enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) were both positive at the time of enrollment. Any cat with discordant results had virus isolation (VI) performed and this result determined the FIV status of the cat. Date of initial FIV infection was not known for any of the cats at the time of enrollment; therefore chronicity of infection for the cats in this study was unknown. Any cat testing positive for feline leukemia virus (FeLV) at enrollment was excluded from the study.

3.2.2 Laboratory findings

Cats were examined every 12 months. At enrollment and at each subsequent examination time point, blood was collected and submitted to IDEXX Laboratories (Westbrook, ME) for complete blood count (CBC), plasma biochemistry, and FIV/FeLV ELISA test (SNAP® FIV/FeLV test, IDEXX Laboratories, Westbrook, ME). Lymphocyte immunophenotyping was performed at IDEXX Laboratories (Westbrook, ME) or Purdue University (West Lafayette, IN). Lymphocyte

immunophenotyping was performed using the protocol described by Lin et al[22]. PCR for FIV (FIV Real PCR™ Test) was performed at IDEXX Laboratories (West Sacramento, CA) using proprietary primers and methodology. Virus isolation was performed at the University of Glasgow Centre for Virus Research (Glasgow, United Kingdom)[23]. The following laboratory parameters were evaluated in FIV-infected and FIV-uninfected cats: hematocrit (HCT), leukocyte count (WBC), neutrophil count (NEUT), lymphocyte count (LYMPH), blood urea nitrogen (BUN), creatinine (CREA), total protein (TP), albumin (ALB), globulin (GLOB), alkaline phosphatase (AlkP), and alanine aminotransferase (ALT) activities, cholesterol (CHOL), glucose (GLUC), sodium (NA), urine specific gravity (USG), CD4 count, total CD8 count, $CD8\beta^{low}$ count, and CD4:CD8 ratio. Reference intervals for hematological and plasma biochemical markers and lymphocyte immunophenotype are listed in Table 1.

3.2.3 Statistical analysis

Data were analyzed using commercially available statistical software (IBM SPSS for Windows version 22). Descriptive statistics are reported and laboratory parameter values are reported as median and range. General linear mixed models regression (GLMM) was used to assess temporal patterns in laboratory parameters, comparing these values between FIV-infected and FIV-uninfected cats and also between FIV-infected cats living in sanctuary housing and FIV-infected cats living in private homes with <7 cats. GLMM was used to account for repeated measures over time within individual cats. Intraclass correlation coefficient (ICC) was calculated for each laboratory parameter to determine the variation within each cat that contributed to the total variations. Age at enrollment was adjusted in all models. The GLMM analyses were performed separately by three time intervals from enrollment to year 2, year 3, and year 4 in order to investigate potential effects of non-random withdrawal (i.e. due to death) of study cats over time. Within each time interval, only cats that had data at all time points were included in the analysis. Normality for each variable was assessed using the Shapiro-Wilk statistic (>0.85). Mathematical data transformations (e.g. log) were applied to correct parameters with a skewed distribution. Statistical significance is set at $P < 0.05$.

3.3 Results

A total of 174 cats were evaluated at enrollment, with 87 FIV-infected cats (29 female spayed (FS) and 58 male neutered (MN)) and 87 FIV-uninfected cats (30 FS and 57 MN) and the median age of 5 years (range: 1.5 – 11). There were 104 cats available for temporal pattern comparison over the 3 year duration including 43 FIV-infected and 61 FIV-uninfected with a median age of 4

years (range: 1.5 – 10). The number of cats evaluated at each interval decreased due to death or dropout of cats for other reasons (e.g. owners moved away or no longer were able to participate). The sample size of cats included for each interval is summarized in Table 2. However, no obvious differences were found in the temporal patterns among the three time intervals. Therefore, only the results of the interval from enrollment to year 2 for overall differences between groups and enrollment to year 3 for temporal change were reported.

The data for the following laboratory parameters were right skewed and log transformed for all statistical analyses: CD4, CD8, CD8β^{low}, CD4:CD8 ratio, WBC, LYMPH, NEUT, BUN, CREA, ALT, ALKP, CHOL, GLU, and TP.

3.3.1 Overall differences in laboratory parameters between FIV-infected and uninfected cats

FIV-infected cats had consistently higher values for CD8β^{low} cells, TP and GLOB compared to FIV-uninfected cats ($P < 0.001$ for each; Figures 1, 2 and 3). The CD4:CD8 ratio was consistently lower in FIV-infected cats compared to FIV-uninfected cats ($P < 0.001$; Figure 4).

3.3.2 Temporal changes in laboratory parameters of FIV-infected cats vs. FIV-uninfected cats

There were statistically significant differences in temporal patterns between FIV-infected and FIV-uninfected cats for CD4, CD8 and LYMPH. CD4 and CD8 values decreased in both FIV-infected and FIV-uninfected cats overtime, but the loss of cells was greater in FIV-infected cats (Figures 5 and 6). LYMPH values decreased in FIV-infected cats but remained stable over time in FIV-uninfected cats (Figure 7).

For FIV-infected cats, compared to FIV-uninfected cats, statistically significant differences in temporal patterns were found for HCT, ALB, and BUN although the overall change was small. Median and range laboratory parameters and P-values are summarized in Table 3. The FIV-infected cats showed a decreasing temporal pattern in HCT values while an increasing pattern was observed in FIV-uninfected cats. Temporal patterns for ALB showed a decreasing pattern for FIV-infected cats whereas values remained stable in FIV-uninfected cats. BUN values were lower in FIV-infected cats and showed a deceasing pattern compared to stable values in the FIV-uninfected cats.

There were no statistically significant differences between FIV infected and uninfected cats for the following laboratory parameters: WBC, ALT, ALKP, GLU, NA and USG.

Intraclass correlation coefficient percentages for laboratory parameters with statistically significant temporal changes ranged from 43 – 96%. BUN had the lowest ICC percentage and CHOL had the highest ICC percentage (data not shown).

3.3.3 Overall differences in laboratory parameters between FIV-infected cats in sanctuary housing and FIV-infected cats

FIV-infected cats had consistently higher laboratory parameter values for GLOB ($P = 0.002$) and NEUT ($P = 0.019$) compared to FIV-infected cats living in private homes (Figures 8 and 9). Laboratory parameter values were consistently lower for ALB ($P < 0.001$) and CHOL ($P = 0.001$) in sanctuary housed cats compared to cats in private homes (Figures 10 and 11).

3.3.4 Temporal changes for laboratory parameters of FIV-infected cats in private homes vs. FIV-infected cats in sanctuary housing

There were statistically significant differences in temporal patterns for the following laboratory parameters: CREA, HCT, WBC, TP, BUN and ALT in FIV-infected cats in sanctuary housing compared to FIV-infected cats in private homes, although the overall change was small. Laboratory parameter values and P-values are summarized in Table 3.

CREA values for FIV-infected cats living in sanctuary housing were lower and decreased over time compared to FIV-infected cats in private homes for which values remained stable. For HCT, a decreasing temporal pattern and lower values were observed for FIV-infected sanctuary housed cats compared to an increasing pattern for FIV-infected cats in private homes. For FIV-infected cats in sanctuary housing, the BUN pattern decreased over time and values were lower compared to FIV-infected cats in private homes for which values remained stable. Laboratory parameter values for ALT showed an increasing pattern for FIV-infected cats in sanctuary housing compared to a decreasing pattern in ALT for FIV-infected cats in private homes.

A difference in temporal pattern for WBC in FIV-infected cats in sanctuary housing compared to FIV-infected cats living in private homes was seen. Median WBC counts and maximum range values were consistently higher in sanctuary housed cats compared to stable values for cats in private homes. TP values showed an increasing pattern for FIV-infected cats in sanctuary housing compared to a decreasing pattern for cats in private homes, although values were higher in sanctuary housed cats.

There were no statistically significant differences in temporal change between the 2 FIV-infected groups for CD4, CD8, and CD8β^{low} counts, CD4:CD8 ratio, LYMPH, ALKP, GLU, NA or USG values (data not shown).

The ICC percentages for markers with statistically significant temporal change comparisons ranged from 42 - 58%. HCT had the lowest ICC percentage and WBC had the highest ICC percentage (data not shown).

3.4 Discussion

The results of this study show that cats naturally infected with FIV had differences in multiple laboratory parameters, and among these, some parameters had differences in temporal patterns compared to FIV-uninfected cats. FIV-infected cats had consistently higher values for CD8β^{low} cells, globulin levels and total protein levels and lower CD4:CD8 ratio values compared to FIV-uninfected cats. Differences in temporal patterns were seen forCD4 and total CD8 T cell values, absolute lymphocyte counts, hematocrit, blood urea nitrogen (BUN), cholesterol and albumin in FIV-infected cats compared to FIV-uninfected cats. For FIV-infected cats living in sanctuary housing consistently higher values for globulin levels and absolute neutrophil counts and lower values for albumin and cholesterol levels. Differences in temporal patterns were seen for hematocrit, albumin, BUN, creatinine, absolute white blood cell counts and total protein in FIV-infected cats living sanctuary housing compared to cats in private homes, however, changes were small. The biological importance of small changes in temporal patterns within or just above the reference interval is not known and may be indicative of disease within an individual cat rather than a trend within the group. Furthermore, based on ICC values, there is a high degree of variability within the individual cats of each group, although slightly less variability within the FIV-infected cats.

The temporal pattern in CD4 T cells in FIV-infected cats compared to FIV-uninfected cats was expected. The lower number of CD4 T cells in FIV-infected cats compared to FIV-uninfected cats has been documented in the literature in both experimental and naturally acquired infection[9,10,16,17]. CD4 T cells are the primary target of the virus and loss of these cells occurs in peripheral blood as well as lymphoid tissue, particularly in the gut-associated lymphoid tissue (GALT) [15,24,25]. It was somewhat surprising that CD4 T cell counts of FIV-uninfected cats decreased over time, similar to the FIV-infected cats. Median CD4 T cell counts fell below the low end of the reference interval by year 3 of the study but values for uninfected cats remained within the reference interval (543 – 1820 cells/µL) reported for healthy adult cats[26]. Temporal

patterns for CD8 T cell counts showed a decreasing pattern in both FIV-infected and FIV-uninfected cats, although the pattern was more pronounced in FIV-infected cats but neither group had a median value below the reference interval. FIV-infected cats had higher CD8 T cell counts compared to FIV-uninfected cats, which was not entirely unexpected. The higher CD8 T cell values in FIV-infected cats were hypothesized to be the result of expansion of $CD8\beta^{low}$ cells in FIV-infected cats, which occurs soon after infection and is reported to continue throughout the chronic stage of disease[17]. In this population of cats, the $CD8\beta^{low}$ cells were consistently higher in FIV-infected cats compared to FIV-uninfected cats but failed to show continued expansion over time. As the duration of FIV infection was unknown for the cats in this study, it may be that these cats had reached a plateau in $CD8\beta^{low}$ production, and that is why there was not a significant difference in temporal change for this parameter. Furthermore, it has been postulated that $CD8\beta^{low}$ cells may transform to CD8β-negative ($CD8\beta^{neg}$) cells in the late stages of chronic infection[19] but $CD8\beta^{low}$ cell counts have not been widely documented in experimental infection so it is not known how this population changes over time. Lymphocyte immunophenotyping for $CD8\beta^{neg}$ cells was not performed in this study and therefore any change in this population was not seen.

In two longitudinal studies of experimentally infected cats and uninfected controls, it was shown that absolute CD4 T cell counts fluctuate over time in both FIV-infected and FIV-uninfected cats and at times showed a decreasing trend in the FIV-uninfected cats[10,18]. The intracorrelation coefficient CD4 and CD8 T cell percentages for the cats in this study support large variation for these values within cats. It has been reported that in healthy cats there is a loss of absolute CD4 and total CD8 T cells in peripheral blood of senior cats (10-14 years old) compared to adult cats (2-5 years old) that is similar to what is seen in aging people and mice[27]. Additionally, a loss of absolute B cells in peripheral blood of senior cats was also reported but interestingly immunoglobulin levels were found to be increased in the senior cats[27]. Immunosenescence, the result of multiple changes to the immune system over time, is associated with loss of leukocytes, lymphocytes and increases in inflammatory cytokines which affect immune response and increase susceptibility to infection and disease in geriatric mammals, although this has not been widely studied in cats[28]. Immunosenescence may have played a role in the loss of T cells and lymphocyte numbers and globulin levels higher than the reference interval in FIV-uninfected cats.

In a recent longitudinal study of experimental FIV infection, FIV infected cats had higher CD8 T cell counts compared to uninfected control cats[10]. Gradual declines in CD8 T cell counts were

noted in half of those FIV-infected cats, whereas the remaining FIV-infected cats appeared to have stable counts. CD8 T cell counts of uninfected control cats appeared stable over time[10]. However, the authors reported no statistical difference in absolute CD8 T cell counts between FIV-infected and uninfected cats, although a higher percentage of lymphocytes expressed the CD8 marker in FIV-infected cats compared to FIV-uninfected cats[10]. The $CD8\beta^{low}$ cell population was not reported for that study[10]. $CD8\beta^{low}$ cell populations have been reported to comprise up to 70% of CD8 T cells ≥7 years post-FIV infection[19,29] As experimentally infected cats are kept in SPF housing, it is not known how other viral pathogens may impact CD4 and CD8 T cell findings. Consistent with the decrease in CD4 and total CD8 T cell counts, lymphocyte numbers decreased over time in both FIV-infected and FIV-uninfected cats, although the decrease was greater in FIV-infected cats. As CD4 and CD8 T cells are a large portion of the lymphocyte population this finding is not unexpected.

The temporal change and decreasing hematocrit in FIV-infected cats compared to FIV-uninfected and FIV-infected cats living in sanctuary housing compared to those in private homes, although mild, may reflect an early change in red blood cell counts associated chronic inflammatory disease. However, in each group the values remained within the reference interval making further biological interpretation of the results difficult. The differences in temporal pattern for albumin between FIV-infected cats and FIV-uninfected cats, and the lower albumin levels in sanctuary housed cats compared to cats in private homes, may be a result of inflammation associated with chronic disease in FIV infection. Chronic inflammation is associated with a decrease in negative acute phase proteins, such as albumin, and an increase in positive acute phase proteins. The production of pro-inflammatory cytokines can interfere with albumin production. It has been shown that FIV-infected cats have increased levels of pro-inflammatory cytokines in circulation. Loss of albumin can also occur through the kidneys, gastrointestinal tract and skin. However, other laboratory parameters and physical examination findings did not support those as likely reasons for decreasing albumin.

The difference in temporal pattern for white blood cell counts between FIV-infected cats in sanctuary housing and cats in private homes and the higher median and maximal range WBC values for sanctuary housed cats raises the question of whether environmental conditions play a role in FIV disease progression. It has been reported that cats living within sanctuary housing have a higher prevalence of upper respiratory infections with pathogens such as feline herpesvirus-1 (FHV-1), feline calicivirus (FCV), *Bordetella bronchiseptica*, *Chlamydophila felis*

and *Mycoplasma* disease compared to those in private homes. Although identifying environmental pathogen exposure was beyond the scope of this study, it is possible that the cats living in sanctuary housing were exposed to an increased number of pathogens, and therefore suffered a higher occurrence of concurrent viral and possible secondary bacterial infection which may have led to increased WBC.

Plasma total protein levels were higher in cats living in sanctuary housing than in cats living in private homes and showed a difference in temporal patterns. The FIV-infected cats in sanctuary housing had consistently higher median and range globulin levels compared to FIV-infected cats in private homes, although a difference in temporal pattern was not found. Increased globulin levels in FIV-infected cats have been previously reported and are thought to be secondary to B cell stimulation and increased immunoglobulin production. Median values for albumin were lower in the sanctuary housed cats compared to those in private homes although no temporal change was found. The higher globulin levels and lower albumin levels could be a consequence of increased pro-inflammatory cytokines that favor humoral immunity and interrupt albumin production, but confirmation of increased cytokine levels was not performed. As duration of infection is unknown in these cats, an increasing trend in the change of globulin levels may have been masked due to the likely varied durations of infection. Additionally, a potential difference in pathogen exposure between sanctuary housed cats and cats in private homes causing a pro-inflammatory response should be considered. This is further supported by increased WBC and absolute neutrophil counts in sanctuary housed cats compared to cats in private homes.

Differences in temporal patterns for blood urea nitrogen (BUN) and creatinine were found in FIV-infected cats compared to FIV-uninfected cats and in FIV-infected cats in sanctuary housing compared to those in private homes. Additionally, a difference in temporal pattern for cholesterol in FIV-infected cats compared to uninfected cats and alanine aminotransferase (ALT) in FIV-infected cats in sanctuary housing compared to FIV-infected cats in private homes. The changes in BUN, creatinine, cholesterol and ALT were small and the median values of each remained within reference intervals therefore the biological significance of these changes is not known; changes may be the result of concurrent disease within a specific cat rather than a trend within the group. A recent study showed no significant difference in renal values between FIV-infected and uninfected cats but found an increased urine protein-to-creatinine (UPC) ratio in FIV-infected cats[30]. The UPC ratio was not measured for cats in this study. Diagnostics to assess liver function

or intestinal mucosal integrity were not performed and further inference as to cause of differences in ALT and cholesterol cannot be made.

There were several limitations to this study. Enrollment of cats in the study was ongoing for two years, therefore not all cats were monitored for a period of 4 years; some were only monitored for 2 years. Although cats in both the infected and uninfected groups were lost to follow-up, more FIV-uninfected cats were lost to follow-up. This led to a greater attrition rate than expected over the course of the study and the resulting sample size may not have been large enough to detect significant differences in temporal patterns. Moreover, loss of cats over the duration of the study may have unintentionally favored selection of healthy cats that survived to the end of the study. In addition, the duration of the study may not have been long enough to observe the full impact of change over time in erythrocyte, leukocyte or lymphocyte subset populations or in albumin and globulin levels. The lack of an FIV-uninfected sanctuary housed population limited a more thorough investigation into the impact of environment on laboratory parameters. Virus isolation was not performed on all cats enrolled in the study and without the results of this test, considered the reference standard, there is a possibility that infection status was not correct in all cats. Finally, as the cats in this study were all naturally infected, and acquired from shelters with limited knowledge of any previous health history, there was no known duration of infection for direct comparison between the cats. Similarly, the age of the cats was an estimate made at the time of entry into the shelter and may not have been accurate, although adjustment for age was performed in the statistical analysis.

3.5 Conclusion

The results of this study showed that FIV-infected cats have higher values for absolute $CD8\beta^{low}$ cell counts, total protein and globulin levels and lower CD4:CD8 ratio compared to uninfected cats. Additionally, differences in temporal patterns were seen for CD4 and total CD8 T cell values, absolute lymphocyte cell counts, hematocrit, BUN, creatinine and albumin levels compared to FIV-uninfected cats. Furthermore, FIV-infected cats in sanctuary housing had higher values for globulin levels and absolute neutrophil counts compared to FIV-infected cats living in private homes. FIV-infected cats in sanctuary housing showed differences in temporal patterns for WBC count, total protein, hematocrit, BUN, creatinine and alanine aminotransferase activity levels compared to cats that lived in private homes. Differences in laboratory parameter values and temporal patterns associated with inflammation and immune activity were seen in FIV-infected cats compared to uninfected cats and FIV-infected cats in sanctuary housing compared to

cats that lived in private homes. These findings are consistent with those reported for experimental infection but raised the question of environmental impact on inflammation and immune response in the progression of disease in FIV infection. Particularly when inflammation and immune activation are two prominent aspects of FIV infection associated with disease progression and may be influenced by increased exposure to pathogens within the environment. Due to the small number of cats in this study and lack of an FIV-uninfected sanctuary housed control group, the results of this study are considered exploratory and cannot be extrapolated to the larger FIV-infected population. Further investigation into the potential impact of living environment on chronic FIV infection is warranted and should be pursued. At this time, CD4 and total CD8 T cell counts may be the most sensitive parameters by which to measure disease progression over the long duration of chronic infection. However, further efforts to define disease progression in FIV infection should include discovery of new biological markers that more accurately reflect the chronic inflammation and immune activation associated with FIV infection, rather than continued investigation into routine laboratory health parameters which may not be specific to FIV infection.

Table 3.1. Reference intervals for hematological, biochemical and lymphocyte immunophenotype markers

[a] Reference intervals as used by Purdue University College of Veterinary Medicine

[b] Dean GA et al. *Veterinary Immunology and Immunopathology*, 28 (1991) 327-335

[c] Willett BJ et al *Immunology*, 78 (1993) 1-6

Marker	Reference interval	Marker	Reference Interval
HCT[a]	30.0 - 45.0%	**BUN**[a]	15 - 35 mg/dL
WBC[a]	6.0 - 18.0 x10³/uL	**CREA**[a]	0.9 - 2.3 mg/dL
NEUT[a]	3.0 - 12.0 x10³/uL	**TP**[a]	5.5 - 7.1 g/dL
LYMPH[a]	1.50 - 7.0 x10³/uL	**ALB**[a]	2.7 - 3.9 g/dL
CD4[b]	543 - 1820 x10³/uL	**GLOB**[a]	2.3 - 3.8 g/dL
CD8[b]	353 - 994 x10³/uL	**ALKP**[a]	23 - 107 U/L
CD8β[low, c]	97 – 219 x10³/uL	**ALT**[a]	20 - 108 U/L
CD4:CD8 ratio	1.2 – 2.6	**CHOL**[a]	45 - 274 mg/dL
		GLU[a]	75 - 134 mg/dL
		NA[a]	150 - 165 mmol/L

Table 3.2. Sample size for groups at each time interval

Groups (n)	Followed 2 years	Followed 3 years	Followed 4 years
FIV -	72	63	34
FIV+	51	43	26
FIV+ private home	32	28	13
FIV+ sanctuary	19	15	13

Table 3.3. Descriptive statistics (median and range) and P-values for selected laboratory parameters over 3 years for FIV-infected and FIV-uninfected cats

Parameter median (range)	Group	Enrollment	Year 1	Year 2	Year 3	P-value
HCT	FIV-	43 (34 – 62)	45 (33 – 62)	45 (28 – 55)	78 (18 – 63)	0.016
	FIV+	42 (21 – 50)	44 (32 – 52)	44 (33 – 52)	44 (27 – 55)	
BUN	FIV-	23 (14 – 38)	24 (17 – 32)	23 (15 – 52)	24 (15 – 41)	0.001
	FIV+	22 (12 – 34)	20 (13 – 28)	22 (14 – 30)	22 (13 – 32)	
CHOL	FIV-	154 (81 – 304)	161 (93 – 334)	151 (82 – 346)	154 (104 – 332)	0.03
	FIV+	140 (76 – 316)	139 (81 – 303)	136 (73 – 336)	128 (70 – 334)	
ALB	FIV-	3.5 (2.5 – 4.2)	3.5 (2.8 – 4.0)	3.5 (2.5 – 4.5)	3.6 (2.6 – 4.2)	0.015
	FIV+	3.2 (2.5 – 3.9)	3.4 (2.5 – 3.9)	3.3 (2.7 – 3.9)	3.1 (2.6 – 3.9)	

Table 3.4. Descriptive statistics (median and range) and P-values for selected laboratory parameters over 3 years for FIV-infected in sanctuary housing and FIV-infected cats in private homes

Parameter median (range)	Group	Enrollment	Year 1	Year 2	Year 3	P-value
HCT	FIV+ private home	41 (32 – 50)	45 (34 – 52)	46 (33 – 52)	46 (31 – 55)	0.05
	FIV+ sanctuary	42 (21 – 48)	41 (32 – 49)	41 (36 – 50)	40 (27 – 49)	
WBC	FIV+ private home	10.3 (5.2 – 20.9)	11.2 (4.1 – 18.7)	8.3 (3.2 – 12.7)	8.7 (4.3 – 13.2)	<0.001
	FIV+ sanctuary	12.7 (4.5 – 23.7)	11.1 (6.6 – 24.6)	10.1 (6.0 – 26.7)	12.3 (6.9 – 36.0)	
TP	FIV+ private home	7.9 (6.5 – 9.9)	8.1 (6.8 – 9.6)	8.0 (6.7 – 9.2)	7.7 (6.4 – 9.2)	0.004
	FIV+ sanctuary	8.0 (7.2 – 8.8)	8.1 (6.8 – 8.8)	8.2 (6.8 – 11.0)	8.2 (7.1 – 10.3)	
BUN	FIV+ private home	23 (14 – 34)	22 (14 – 28)	24 (15 – 30)	23 (13 – 32)	0.03
	FIV+ sanctuary	20 (12 – 28)	18 (13 – 22)	18 (14 – 23)	20 (14 – 29)	
CREA	FIV+ private home	1.4 (0.9 – 2.0)	1.5 (1.0 – 1.9)	1.5 (1.0 – 1.9)	1.5 (1.1 – 2.2)	<0.001
	FIV+ sanctuary	1.4 (1.0 – 2.1)	1.2 (0.6 – 1.8)	1.3 (0.9 – 1.8)	0.9 (1.7 – 2.5)	
ALT	FIV+ private home	51 (10 – 587)	52 (15 – 239)	50 (27 – 197)	52 (29 – 92)	0.007
	FIV+ sanctuary	50 (26 – 82)	47 (14 – 115)	46 (12 – 96)	59 (41 – 78)	

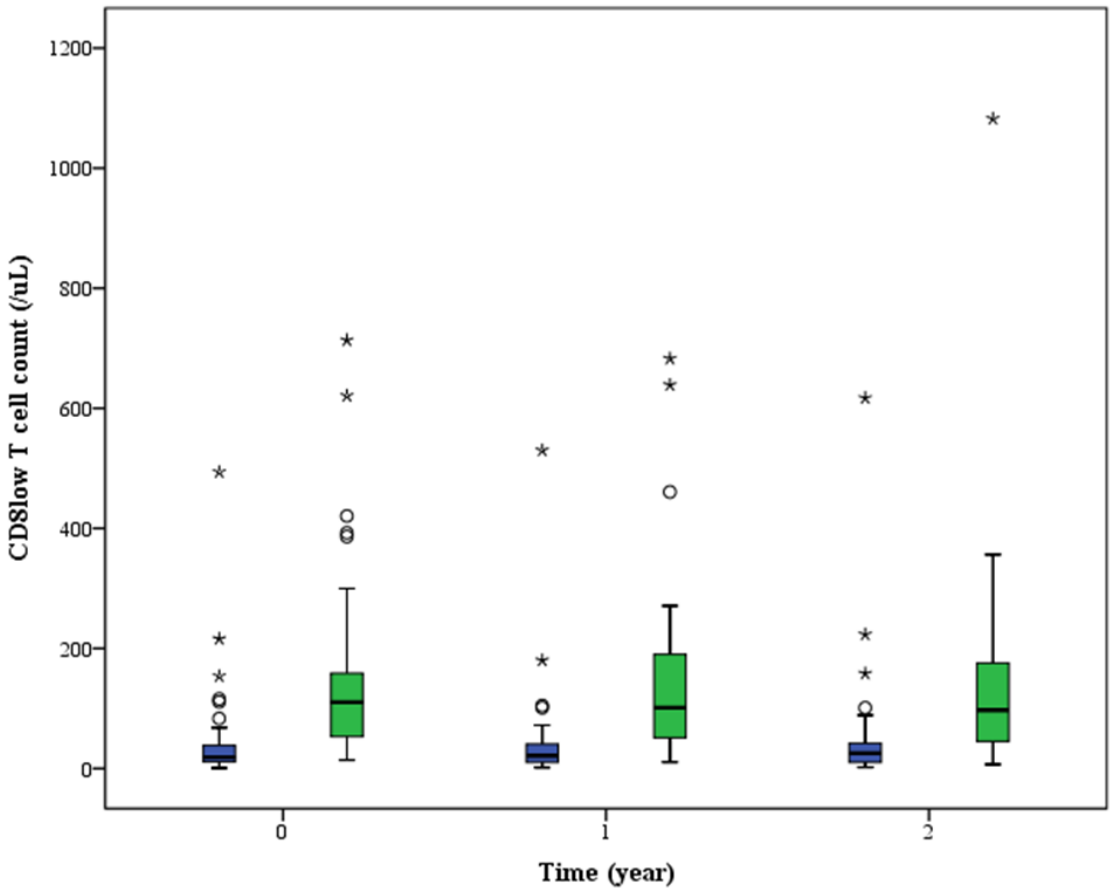

Figure 3.1. Descriptive statistics (median and range) for differences in absolute CD8β^{low} T cell counts for FIV-infected and FIV-uninfected cats over 2 years

Blue boxes: FIV-uninfected cats; Green boxes: FIV-infected cats, Open circles: extreme values; Stars: outliers; P <0.001.

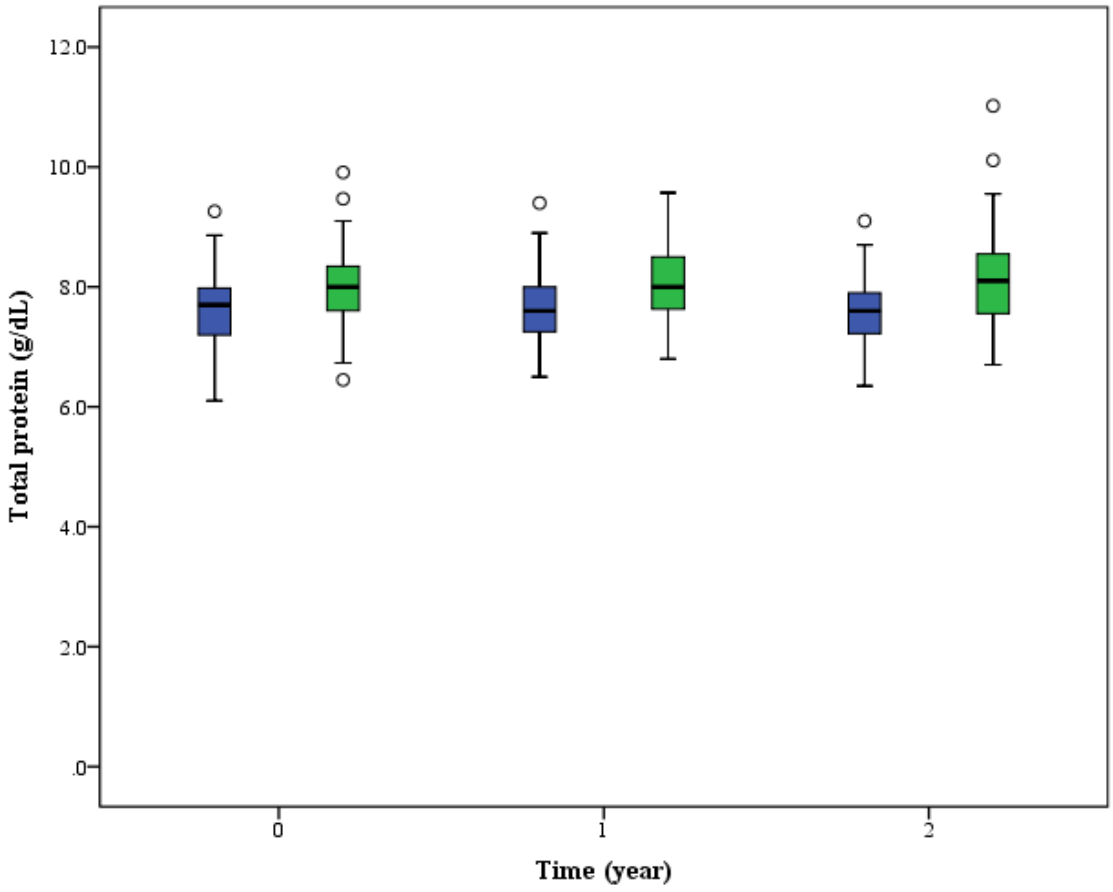

Figure 3.2. Descriptive statistics (median and range) for differences in total protein for FIV-infected and uninfected cats over 2 year

Blue boxes: FIV-uninfected cats; Green boxes: FIV-infected cats, Open circles: extreme values; Stars: outliers; P <0.001.

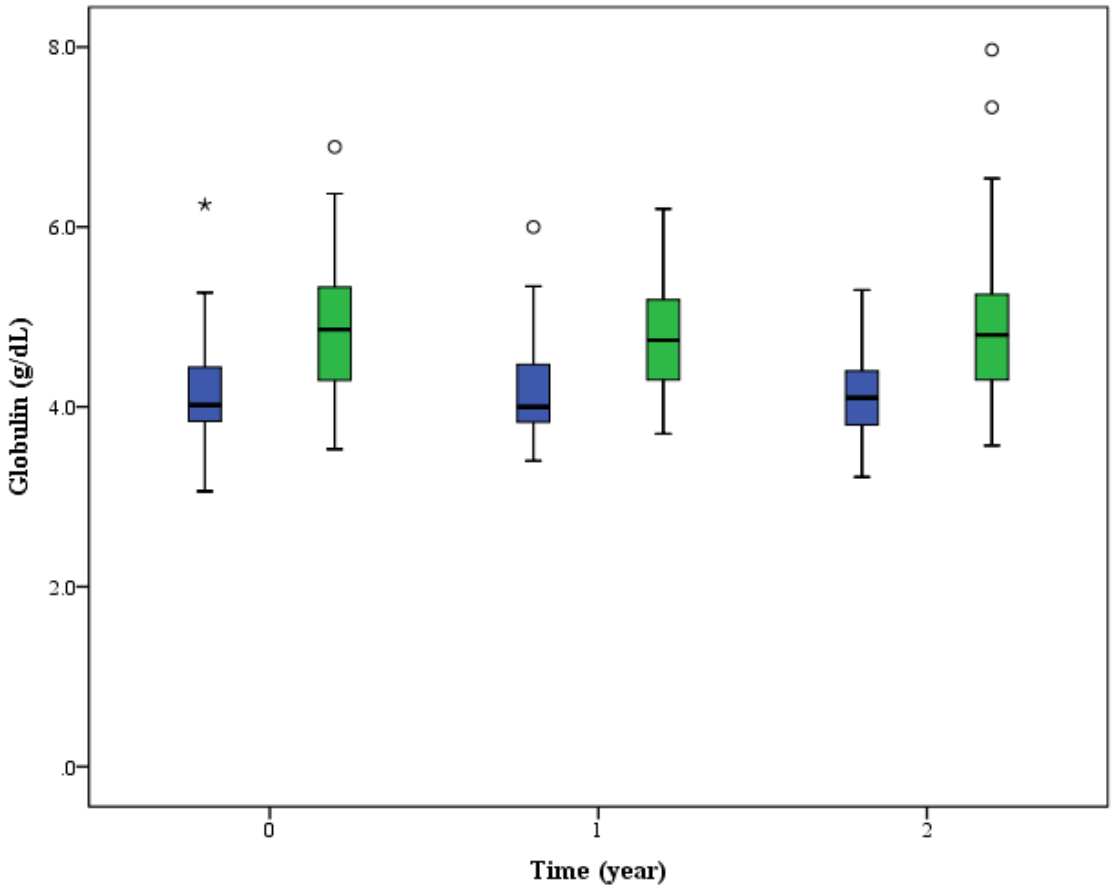

Figure 3.3. Descriptive statistics (median and range) for differences in globulin levels for FIV-infected and uninfected cats over 2 years

Blue boxes: FIV-uninfected cats; Green boxes: FIV-infected cats, Open circles: extreme values; Stars: outliers; P <0.001.

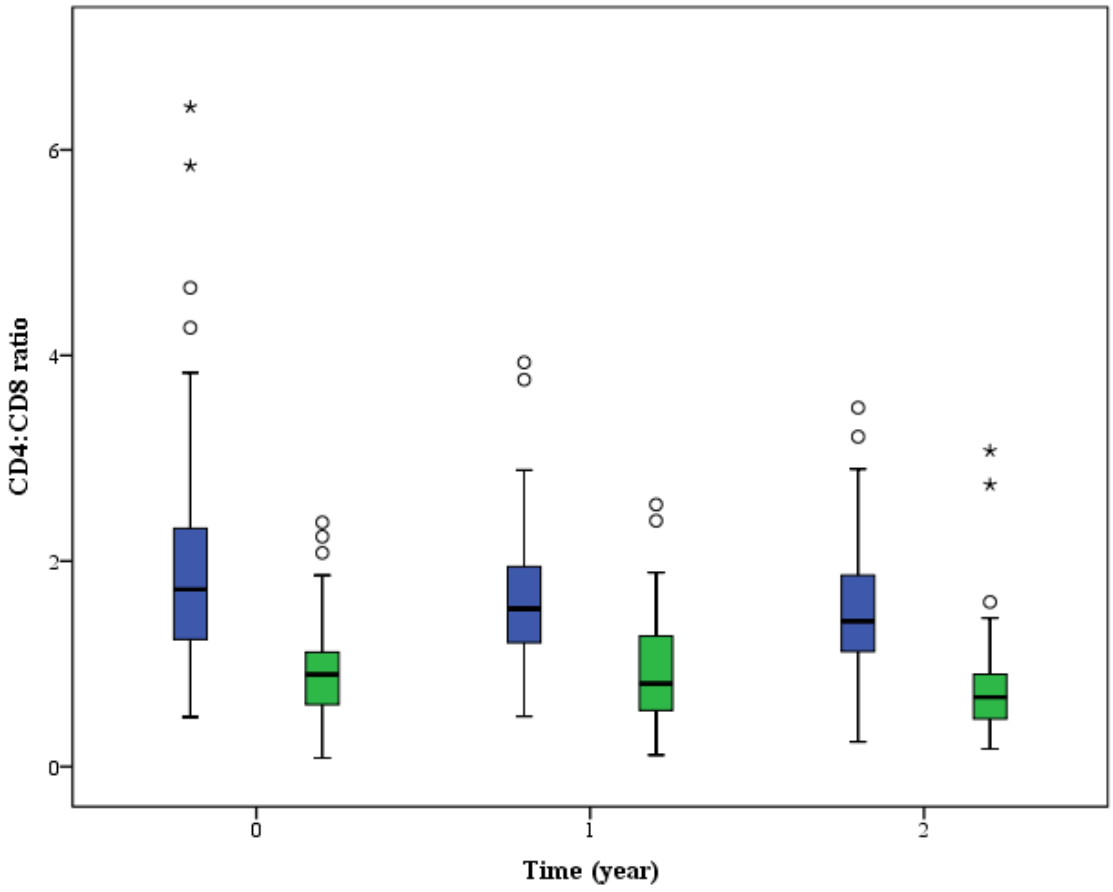

Figure 3.4. Descriptive statistics (median and range) for differences in CD4:CD8 ratio for FIV-infected and uninfected cats over 2 years

Blue boxes: FIV-uninfected cats; Green boxes: FIV-infected cats, Open circles: extreme values; Stars: outliers; P <0.001.

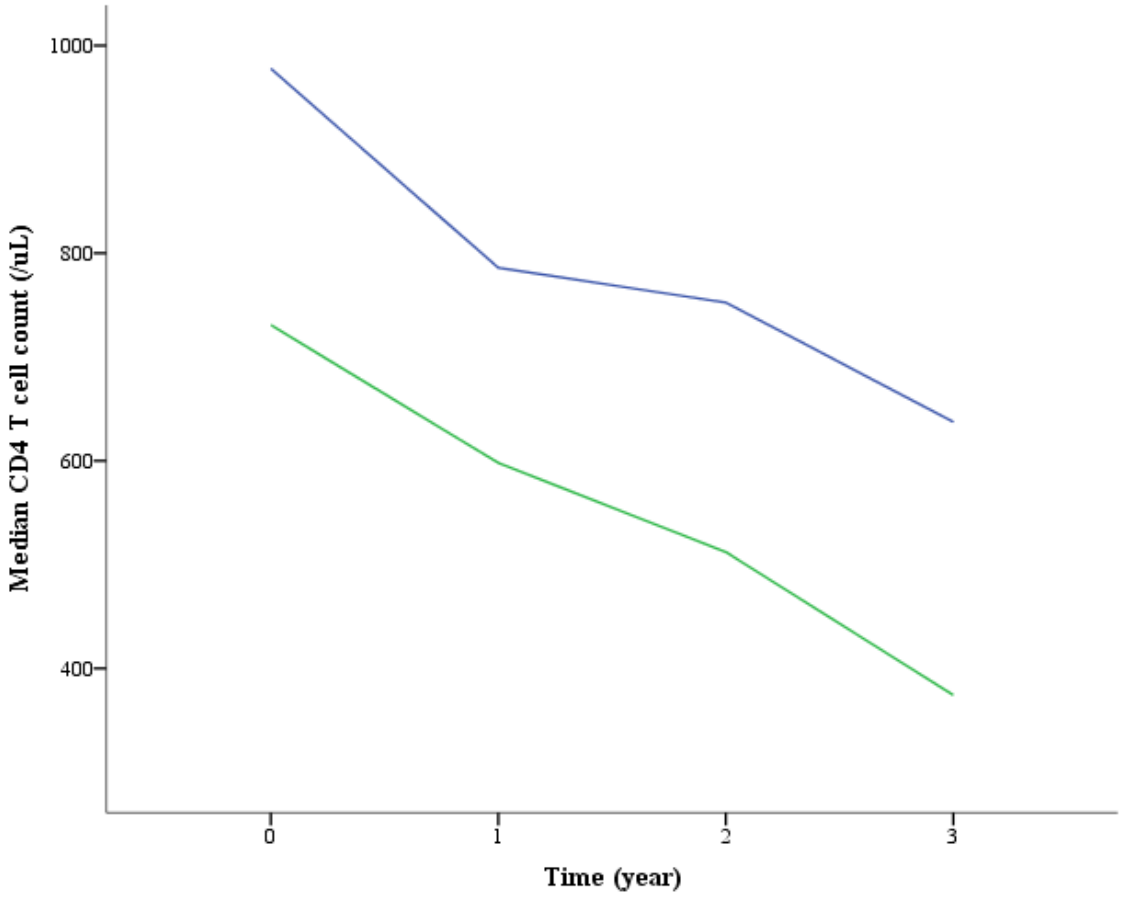

Figure 3.5. Temporal patterns for absolute CD4 T cell counts in FIV-infected and uninfected cats over 3 years

Blue lines: FIV-uninfected cats; Green line: FIV-infected cats; P = 0.008

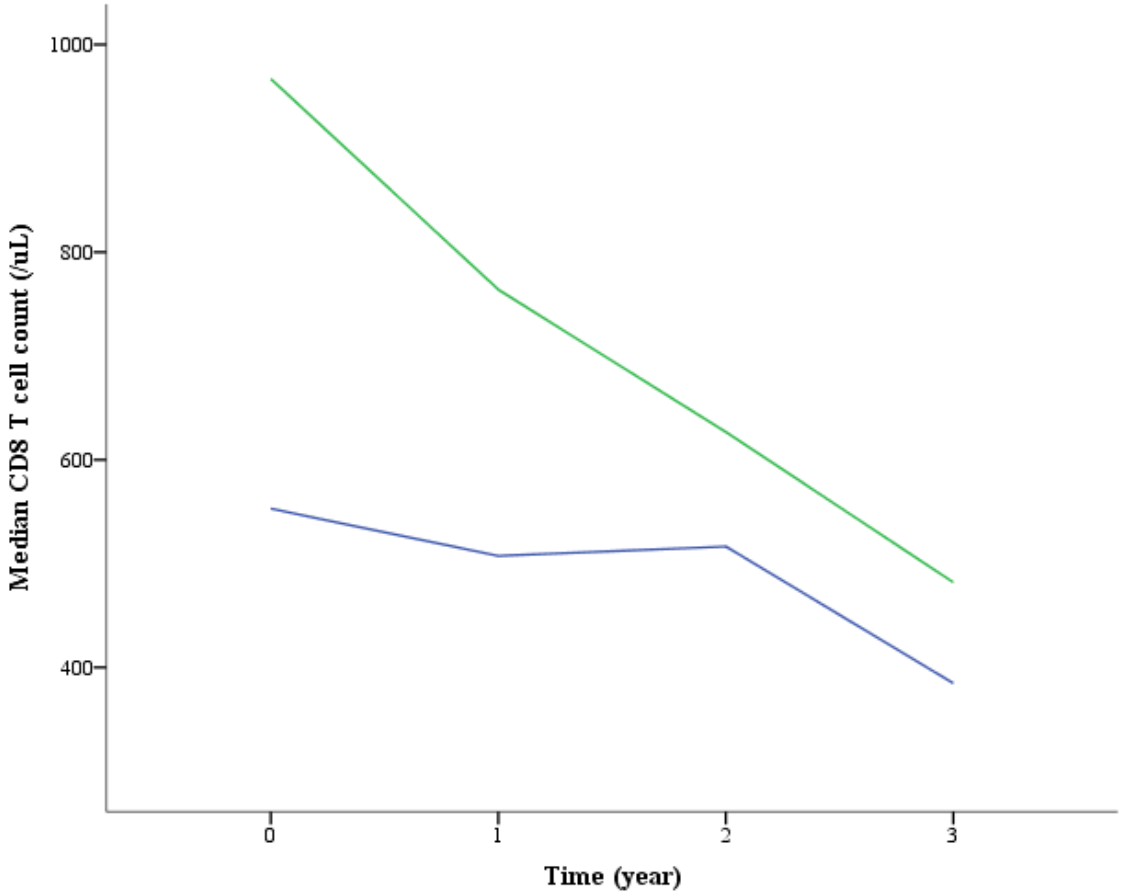

Figure 3.6. Temporal patterns for absolute CD8 T cell counts in FIV-infected and uninfected cats over 3 years

Blue line: FIV-uninfected cats; Green line: FIV-infected cats; P = 0.01

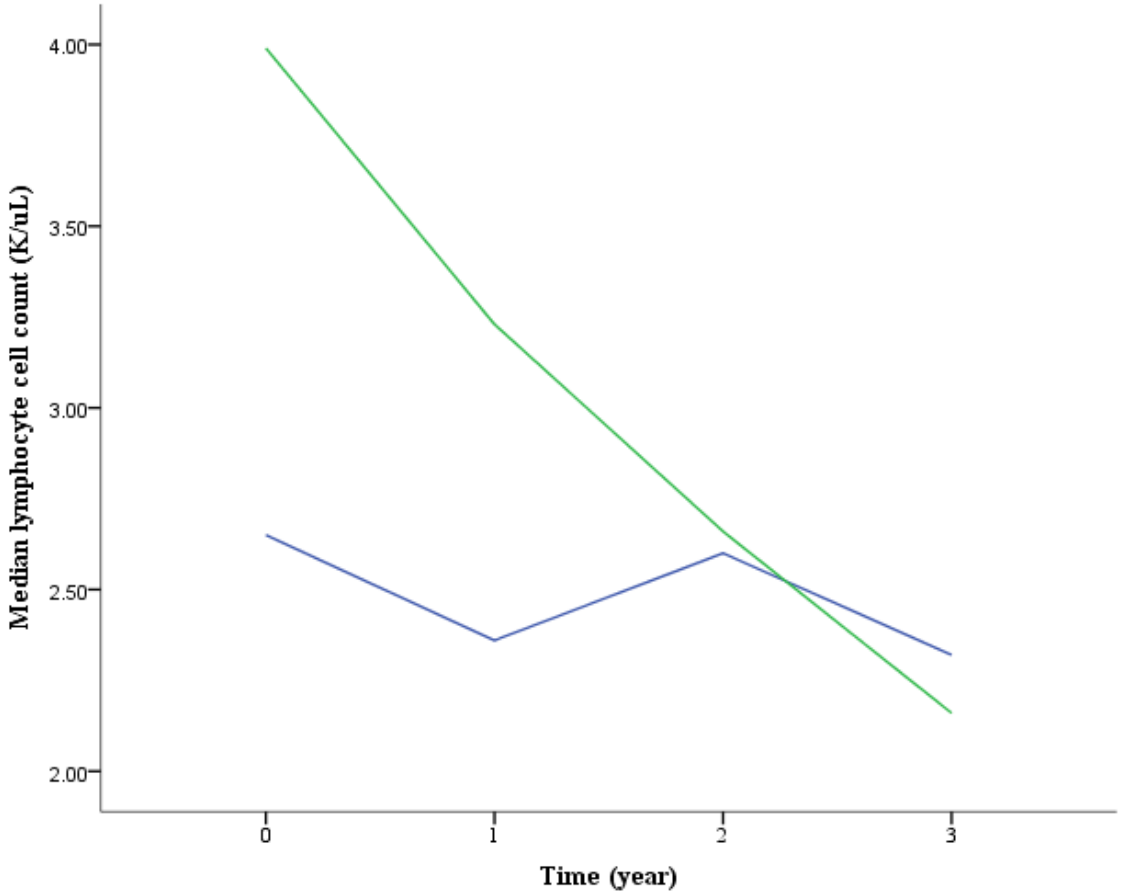

Figure 3.7. Temporal patterns for absolute lymphocyte counts in FIV-infected and uninfected cats over 3 years

Blue line: FIV-uninfected cats; Green line: FIV-infected cats; P = 0.01

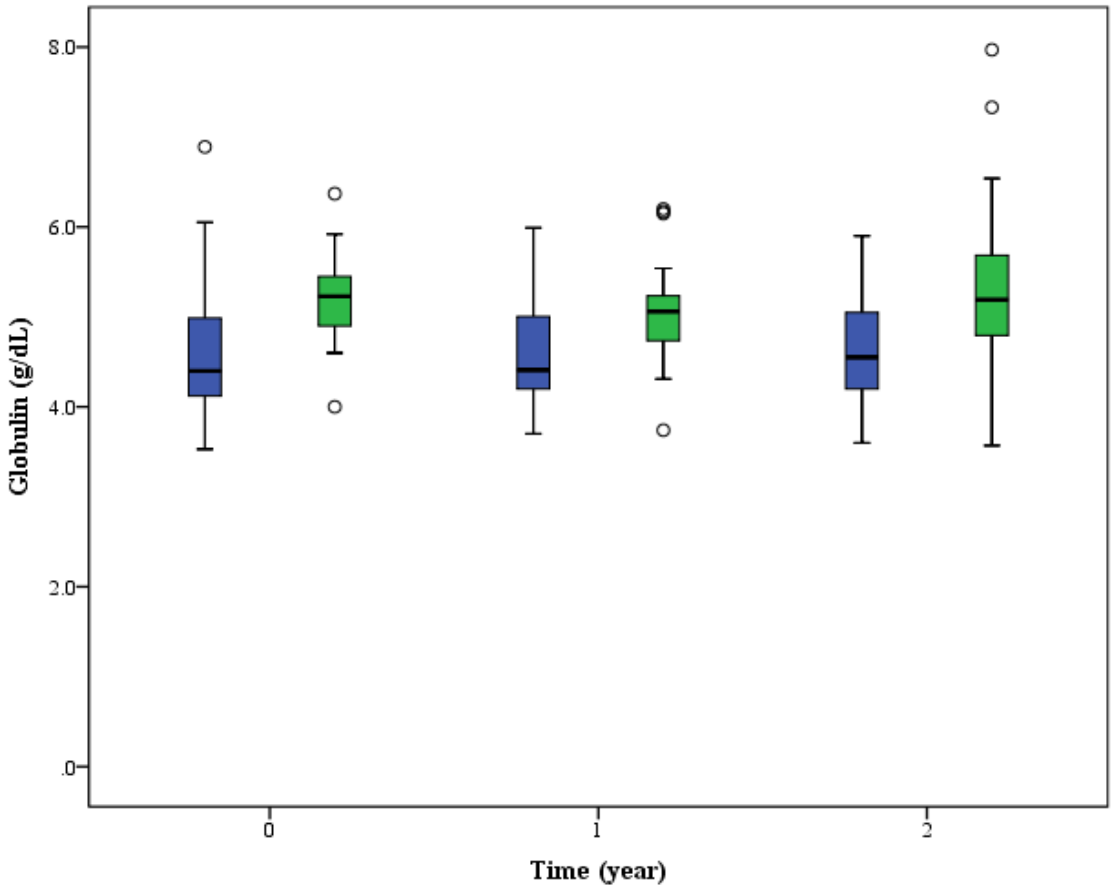

Figure 3.8. Descriptive statistics (median and range) for differences in globulin levels for FIV-infected cats in sanctuary housing and FIV-infected cats in private homes over 2 years

Blue boxes: FIV-infected cats in private homes; Green boxes: FIV-infected cats in sanctuary housing, Open circles: extreme values; Stars: outliers; P = 0.002.

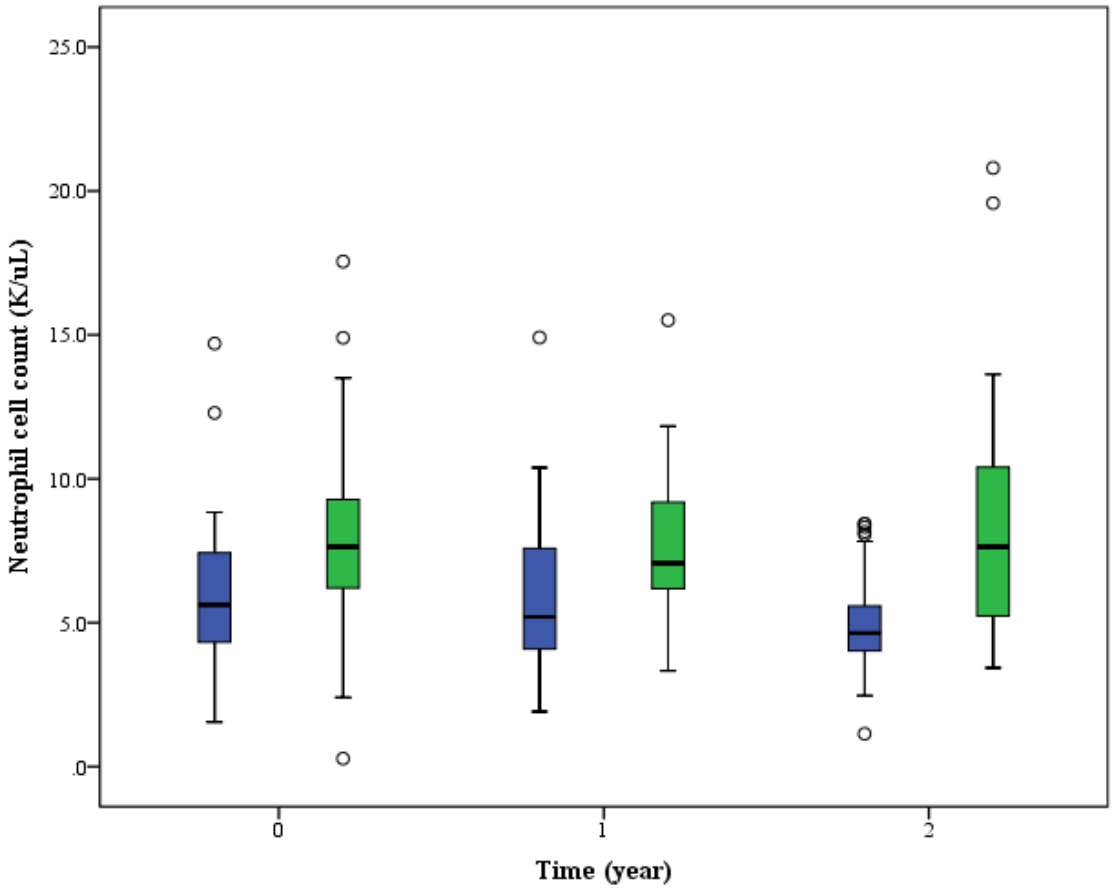

Figure 3.9. Descriptive statistics (median and range) for differences in absolute neutrophil counts for FIV-infected cats in sanctuary housing and FIV-infected cats in private homes over 2 years

Blue boxes: FIV-infected cats in private homes; Green boxes: FIV-infected cats in sanctuary housing, Open circles: extreme values; Stars: outliers; P = 0.02.

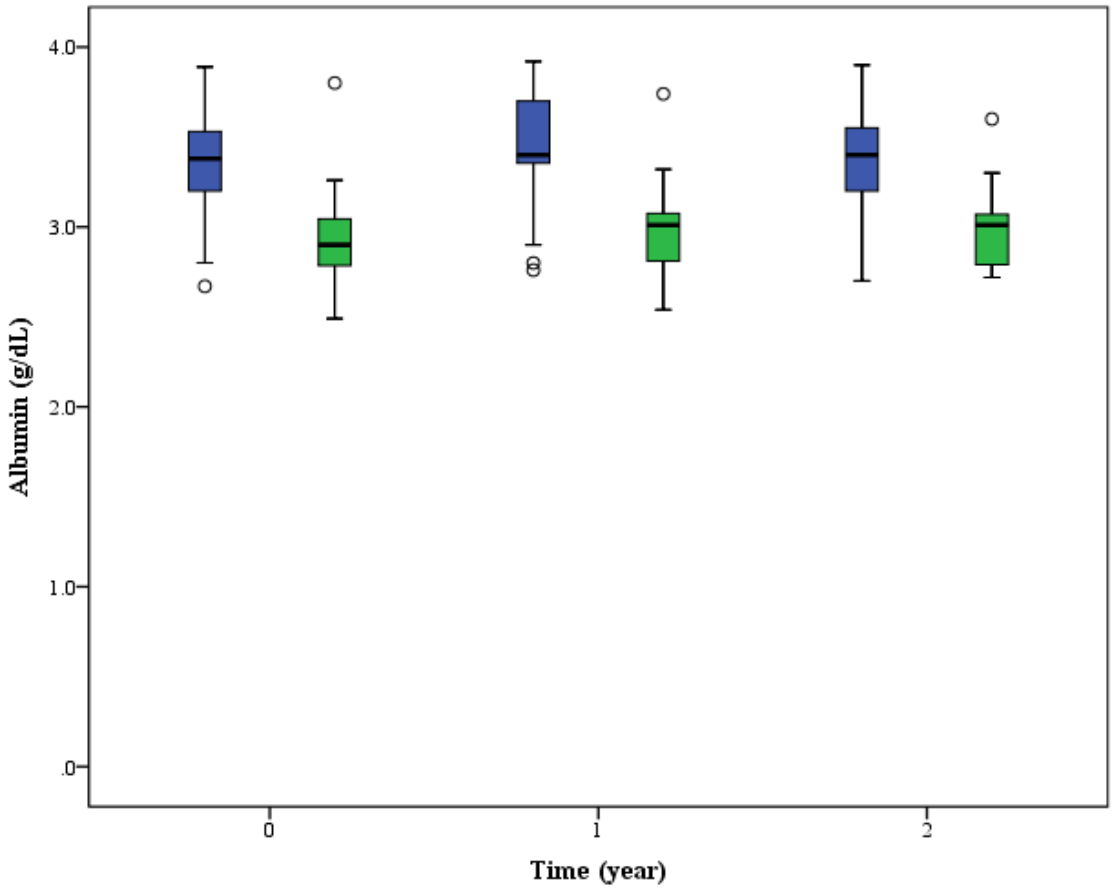

Figure 3.10. Descriptive statistics (median and range) for differences in albumin levels for FIV-infected cats in sanctuary housing and FIV-infected cats in private homes over 2 years

Blue boxes: FIV-infected cats in private homes; Green boxes: FIV-infected cats in sanctuary housing, Open circles: extreme values; Stars: outliers; P <0.001.

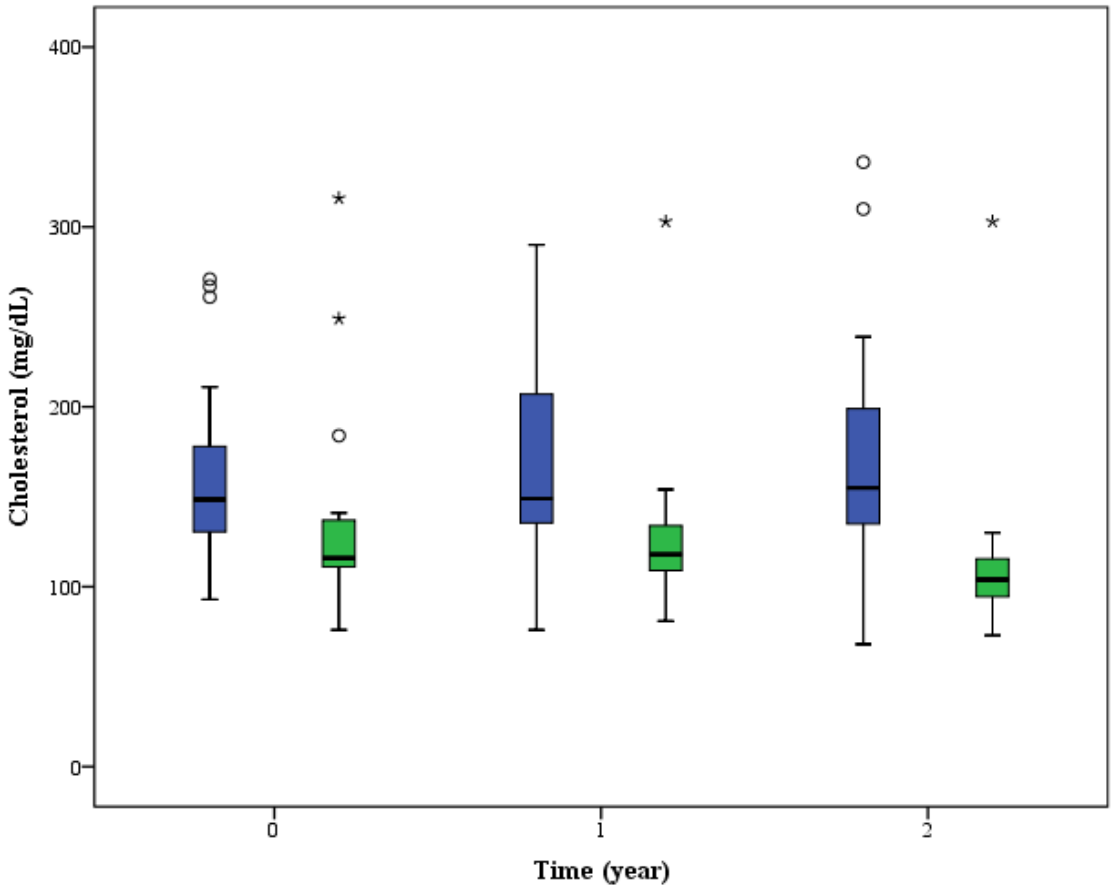

Figure 3.11. Descriptive statistics (median and range) for differences in cholesterol levels for FIV-infected cats in sanctuary housing and FIV-infected cats in private homes over 2 years

Blue boxes: FIV-infected cats in private homes; Green boxes: FIV-infected cats in sanctuary housing, Open circles: extreme values; Stars: outliers; P = 0.001.

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CHAPTER 4. ILLNESS AND MORTALITY RATES IN CATS NATURALLY INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS AND MARKERS ASSOCIATED WITH THESE OUTCOMES

4.1 Introduction

Feline immunodeficiency virus (FIV) is an important life-long lentiviral infection in cats which leads to immunosuppression and increased risk of certain opportunistic infections, neoplasias and early mortality. Prevalence of FIV is estimated to be approximately 2.5% of owned cats in the United States however, prevalence is increased in cats allowed outdoors (4.3%), those living in large shelters where environments are consistent with hoarding situations (8.8%), and in cats presenting to a veterinarian with clinical signs of illness (up to 20.6%) [1,2]. Male, intact, outdoor cats are at greatest risk of infection due to territorial fighting behavior[1,3,4].

In FIV infection, disease course is typically categorized into three stages: 1) acute infection, 2) asymptomatic or chronic infection and 3) symptomatic or end stage infection. Acute infection may be characterized by transient signs of illness including lethargy, fever, lymphadenopathy and diarrhea that occur several weeks post-infection[5]. During this period there is a decrease in CD4 T cell counts, an increase in CD8 T cell counts due to expansion of the $CD8\beta^{low}$ subset, and an inversion of the CD4:CD8 ratio[5-8]. The expansion of $CD8\beta^{low}$ cells, unique to FIV-infected cats, are reported to have anti-FIV properties that aid is controlling viremia but an exact mechanism of action is unknown[6,7,9]. As cats transition into the chronic phase of infection CD4 T cells are restored but not to pre-infection levels. Chronic infection lasts for a variable period of years during which patients show no outward signs of clinical illness on physical examination[10]. There is a slow decline in CD4 T cell counts, a continued expansion of $CD8\beta^{low}$ cells, and continued decrease in the CD4:CD8 ratio[6,8,11]. Hypergammaglobulinemia is often reported in the chronic stage of FIV infection and is reported to be a polyclonal gammopathy secondary to FIV-related B cell stimulation. In a recent study it was reported that as cats progress to the symptomatic stage of infection plasma globulin levels are further increased compared to the asymptomatic stage of

infection[12]. Cats progressing to end stage immunodeficiency syndrome typically suffer loss of body condition, muscle wasting, treatment-refractory opportunistic infections, anemia, leukopenia, lymphopenia, neoplasia and death[13]. It is anticipated cats will have a marked decline in CD4 T cells and CD4:CD8 ratio, anemia and leukopenia however, clinical pathological and lymphocyte immunophenotype data in end stage disease has not been well reported. The knowledge of biochemical and lymphocyte immunophenotype changes in cats with end stage disease is still sparse as there have been few studies performed and reported results are based on only a few cats which may not reflect end stage disease in the larger population of cats infected with FIV[14,15].

In FIV infection, there are no distinct biomarker guidelines to stage disease or determine increasing risk of illness or progression to end stage disease. The study of FIV infection presents several unique challenges: 1) the lengthy duration of the asymptomatic chronic phase of infection makes consistent follow-up difficult and expensive; 2) it is not known what percentage of FIV-infected cats will succumb to FIV compared to other diseases unrelated to FIV infection; and 3) it is not known what factor or factors are definitively associated with progression from asymptomatic to symptomatic or end stage disease[8,16].Current recommendations for monitoring FIV-infected cats in the asymptomatic phase of infection are twice yearly physical examination and once yearly routine hematological and serum biochemistry panels to assess for abnormalities.[17,18] While important for monitoring health, these recommendations only identify disease once it has occurred and do not evaluate immune parameters, such as CD4 and CD8 T cell counts or viral loads, that may identify FIV disease progression prior to the occurrence of clinical signs of illness or death.

In this study, it was hypothesized that: 1) FIV-infected cats have an increased incidence of illness and mortality rate compared to FIV-uninfected cats; 2) FIV-infected cats have increased markers of inflammation and immune activity compared to FIV-uninfected cats and; 3) FIV-infected cats residing sanctuary housing will have an increased incidence of illness, mortality and inflammation compared to FIV-infected cats living in private homes. The aim of this prospective study was to determine the incidence of indicators of illness on physical examination and mortality rate in3 cohorts of cats and evaluate the association of lymphocyte immunophenotype, inflammatory and hepatic markers of disease to the incidence of indicators of illness and mortality rate in these cohorts. Cohorts were comprised of cats naturally infected with FIV residing in either sanctuary housing or private homes (<7 cats) and FIV-uninfected control cats

residing in private homes. The association between living environment and incidence of indicators of illness and mortality rate in FIV-infected cats was also assessed. Percent change in lymphocyte immunophenotype, inflammatory and hepatic markers over the 1-year and 6-month intervals prior to illness and death was evaluated for associations with progression to illness and mortality in FIV-infected cats. The clinical outcome for a subset of 44 FIV-infected cats enrolled in this study has been previously reported for the first 22 months of the study period[19].

4.2 Materials and Methods

4.2.1 Animals

Cats were enrolled from adoption guarantee shelters (PAWS Chicago and Treehouse Humane Society, (Chicago, IL)), the Fitzhugh B. Crews FIV Sanctuary (Memphis, TN) or private homes in the Chicago, IL and Memphis, TN metropolitan areas. Cats were considered FIV-infected when enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) were both positive at the time of enrollment. Any cat with discordant results had virus isolation (VI) performed and this result determined the FIV status. Any cat testing positive for feline leukemia virus (FeLV) at enrollment was excluded from the study. Illness was defined by the following : 1) a body condition score $\leq$3 out of 9[20]; or 2) a gingival score (GS) $\geq$2 out of 3[21] and any degree of gingivostomatits, or tooth root exposure or loose teeth; or 3) nasal or ocular mucopurulent discharge; or 4) neoplasia that was visible or palpable on physical examination and confirmed by histopathology. At enrollment all cats received a physical examination; only cats that were free of evidence of illness as defined above on physical examination were included in this study.

4.2.2 Laboratory findings

FIV-infected cats were examined every 6 months and FIV-uninfected cats were examined every 12 months. At enrollment and at each subsequent examination time point, blood was collected and submitted to IDEXX Laboratories (Westbrook, ME) for complete blood count (CBC), plasma biochemistry, and FIV/FeLV ELISA test (SNAP® FIV/FeLV test, IDEXX Laboratories, Westbrook, ME). Lymphocyte immunophenotyping was performed at IDEXX Laboratories (Westbrook, ME) or Purdue University (West Lafayette, IN). Lymphocyte immunophenotyping was performed using the protocol described by Lin et al (2013)[22]. PCR for FIV (FIV Real PCR™ Test) was performed at IDEXX Laboratories (West Sacramento, CA) using proprietary primers and methodology. VI was performed at the University of Glasgow Centre for Virus Research (Glasgow, United Kingdom)[23]. Necropsies were performed on all cats that died during the study

unless the owner declined. Necropsies were performed at the Indiana Animal Disease Diagnostic Laboratory at Purdue University by one of the authors (TL). If autolysis interfered with the necropsy diagnosis then the cause of death was reported as unknown. Death was considered associated with FIV infection if the histopathological diagnosis included bone marrow hyperplasia, lymphoid hyperplasia, lymphoid hypoplasia or depletion, or lymphadenomegaly, and at least one of the following: lymphoma (at any site), other neoplasia, bacterial tracheitis, bronchitis, rhinitis, or conjunctivitis, lymphocytic-plasmacytic or necrotizing enteritis, or emaciation [5,24-26].

The following were evaluated for association with progression to illness or death: 1) markers of inflammation: hematocrit (HCT), leukocyte count (WBC), neutrophil count (NEUT), lymphocyte count (LYMPH), total protein (TP), albumin (ALB), and globulin (GLOB); 2) markers of T cell immune status and activation: CD4 count, CD8 count, $CD8\beta^{low}$, CD4:CD8 ratio and $CD4{:}CD8\beta^{low}$ ratio; and 3) markers of hepatic inflammation and injury: alkaline phosphatase (AlkP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities, cholesterol (CHOL) and glucose (GLUC).

Changes in the laboratory values were assessed at one year prior to onset of illness or death for FIV-infected and FIV-uninfected cats that developed illness or died during the study. For the cats that did not develop illness or survived to the end of the study, the changes in values were assessed at one year prior to the end of the study. Values at 6-months prior to illness, death, or the end of the study were also evaluated for FIV-infected cats.

4.2.3 Statistical analysis

Data were analyzed using commercially available statistical software (IBM SPSS for Windows version 22). Kaplan-Meier method was used to estimate median time to death and onset of illness in FIV-uninfected cats, FIV-infected cats living in sanctuary housing, and FIV-infected cats living in private households with <7 cats. Cox regression was used to compare hazard rate of mortality and illness between the 3 groups of cats with an adjustment for age at enrollment to address the concern that older cats are more likely to develop illness or die compared to younger cats. Cox regression was also used to assess the association of percent change in lymphocyte immunophenotype, inflammatory and hepatic markers with mortality and illness in FIV-infected cats over a 6-month and 1-year interval prior to death or illness. The percent change in value for analytes was computed using the equation: $(T_0 - T_{-1})/T_{-1} \times 100$. Where T_0 is the sample collected

prior to death or at the time of illness and T_{-1} is the sample collected 1 year or 6 months prior to T_0. Analytes were investigated using Cox regression with a stepwise procedure as groups (immunophenotype panel, inflammatory panel and liver panel) and only the statistically significant ($P < 0.05$) markers within each group were reported. Receiving operator curve (ROC) analysis was performed for CD4 to determine discriminating ability for illness and mortality.

4.3 Results

A total of 174 cats were evaluated and 130 cats (78 FIV-uninfected cats and 52 FIV-infected cats) were included in this study and monitored up to 5 years, depending on enrollment and death dates. Twenty-six FIV-infected cats lived in sanctuary housing of ≥10 cats and 26 lived in private homes of <7 cats. Sixteen FIV-uninfected cats and 3 FIV-infected cats residing in private homes were lost to follow-up. Age of the cats at enrollment was not significantly different among the three groups (P=0.189, ANOVA test) (Table 1).

4.3.1 Kaplan-Meier analysis

The median time to death was 27 months (95% CI 9.5, 44.5) for FIV-infected cats in sanctuary housing. FIV-uninfected cats and FIV-infected cats in private homes did not reach the median time for death during the 5-year follow-up period (Figure 1). Compared to FIV-uninfected cats, FIV-infected cats living in sanctuary housing had an 11.7 (95% CI 4.1, 33.9; P<0.001) fold increase in mortality rate and FIV-infected cats living in private homes had a 4.1 (95% CI 1.2, 14.7; P=0.028) fold increase in mortality rate after adjusting for age at enrollment. FIV-infected cats living in sanctuary housing had a 5.1 (95% CI 1.4, 18.2) fold increase in the rate of mortality associated with FIV compared to those FIV-infected cats living in private homes.

The median time to the onset of illness was 23 months (95% CI 14.7, 31.3) for FIV-infected cats living in sanctuary housing and 53 months (95% CI 37.3, 68.7) for FIV-infected cats living in private homes (Figure 2). Compared to FIV-uninfected cats, FIV-infected cats living in sanctuary housing had a 15.1 (95% CI 6.2, 37.0; P <0.001) fold increase in illness rate and FIV-infected cats living in private homes had a 3.0 (95% CI 1.1, 8.7; P = 0.037) fold increase in illness rate after adjusting for age at enrollment.

Necropsies were performed on 18 of 21 FIV-infected cats and necropsy findings indicated that death was associated with FIV infection in 15 of 26 FIV-infected cats (58%) living in sanctuary housing and 3 of 26 FIV-infected cats (12%) living in private homes. Among 25 FIV-infected cats that developed illness, 15 died and had necropsy findings associated with FIV infection.

There was no difference in mortality rate, following the onset of illness, between FIV-infected cats living in sanctuary housing and those living in private homes, after adjusting for age at enrollment (P=0.600). The median time to death, following onset of illness, in FIV-infected cats with necropsy findings associated with FIV infection was 27 months (95% CI 14.5, 39.5). In cats with necropsy findings associated with FIV, those that developed clinical signs of illness had a 3.8 (95% CI 1.0, 14.1) fold increase in the rate of mortality compared to those cats that did not develop illness (P = 0.05).

4.3.2 Cox regression analysis for mortality rate

In FIV-infected cats, after adjusting for environment and age at enrollment, a greater decrease in TP and ALB in the 1-year interval prior to death was associated with an increased mortality rate (P = 0.042 and 0.009, respectively) in the stepwise survival analysis (Table 2). In the 6-month interval prior to death, a greater decrease in HCT and ALB was associated with an increased mortality rate (Table 2). There were no associations between hepatic markers and mortality in FIV-infected cats.

4.3.3 Cox regression analysis for illness rate

In FIV-infected cats, after adjusting for environment and age at enrollment, a greater decrease in CD4 and ALB (P = 0.002 and 0.039, respectively) and a greater increase in the CD4:CD8 ratio were associated with an increased rate of illness in the 1-year time interval prior to the clinical signs of illness on physical examination in the stepwise survival analysis (Table 2). Over the 6-month time interval prior to the onset of clinical signs of illness in FIV-infected cats, a greater decrease in CD4 (P = 0.041) and a greater increase in NEUT (P = 0.040) was associated with an increased rate of illness (Table 2). There were no associations between hepatic markers and incidence of illness in FIV-infected cats.

There were no associations between inflammatory, immunophenotype, or hepatic markers and incidence of illness or mortality in FIV-uninfected cats at the 1 year interval. ROC analysis for CD4 did not show adequate ability to discriminate for progression to illness or mortality (data not shown).

4.4 Discussion

The results of this study show an increase in the rate of mortality and illness in FIV-infected cats compared to FIV-uninfected cats; furthermore, FIV-infected cats living in sanctuary housing show an increase in rate of mortality and illness compared to FIV-infected cats living in private

homes. In FIV-infected cats, a greater increase in TP and a greater decrease in HCT and ALB prior to death, was associated with an increased mortality rate in the stepwise survival analysis. A greater decrease in CD4and ALB and a greater increase in CD4:CD8 ratio and NEUT prior to the onset of illness was associated with an increased rate of illness in FIV-infected cats. These results suggest that, in FIV-infected cats, mortality rate is associated with chronic inflammation and onset of illness is associated with immune activity. However, given the complicated nature of FIV infection, the inflammatory and immune parameters are undoubtedly intertwined. It has been reported that alterations in cytokine production, activation of T regulatory (T_{reg}) cells, and up-regulation and overexpression of inhibitory T cell surface proteins are associated with immunocompromise and chronic inflammation during FIV infection[27]. Increased circulating pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-1, IL-10, and tumor necrosis factor alpha (TNF-α) and decreased circulating IL-12 resulted in an immune response that favored humoral (antibody) immunity and decreased cell-mediated immunity, leaving the host unable to mount an appropriate immune response to pathogens, particularly to intracellular pathogens[28-30]. T_{reg} cell activation limits the amount of IL-2 produced; a key cytokine produced by T cells to propagate an adaptive immune response to pathogens[31,32]. Up-regulation and overexpression of surface proteins, CTLA-4 and B7.1, on activated T cells act as co-stimulatory signals between T cells and induce a state of anergy (immune inactivity) [33-35]. The combination of these factors may create a paradoxical situation, in which, immune activation responds to inflammatory signals but stimulates dampening of the adaptive immune response, rather than proliferation of a protective response.

The definition for clinical signs of illness were chosen based on previous studies that reported FIV-infected cats have a higher risk of upper respiratory infections, dental disease, loss of body condition and neoplasia compared to FIV-uninfected cats[10,13,36,37]. The results of this study further support that FIV-infected cats have an increased rate of illness characterized by loss of body condition and respiratory and oral disease and neoplasia. Some clinical signs included in criteria for clinical signs of illness, such as dental disease and neoplasia, may not be readily apparent to a lay person. Therefore, it remains important for owners to follow current retroviral guidelines for FIV-infected cats to be examined twice yearly regardless of health status of a cat[17,18].

An increased rate of mortality and illness observed in the FIV-infected cats living in sanctuary housing compared to FIV-infected cats in private homes is not entirely unexpected. Identifying an underlying cause of illness in enrolled cats was beyond the scope of this study however, recent publications in shelter medicine have reported increased illness, particularly upper respiratory infection (URI), among cats living in shelter environments [38-41]. Although certain upper respiratory tract pathogens are almost ubiquitous in the cat population, several studies have reported an increased prevalence of feline herpesvirus-1 (FHV-1), feline calicivirus (FCV), *Bordetella bronchiseptica*, *Chlamydophila felis* and *Mycoplasma* species within shelter settings[38-41]. Several guidelines have made recommendations for care and management of large multi-cat housing situations and efficient use of resources including medical management, quarantine and isolation to limit pathogen exposure, reduction of fomite exposure via personnel, and adequate housing facilities (including space and ventilation) to reduce incidence of infectious diseases[42,43]. In addition to basic housing construction requirements, alleviation of environmental and inter-cat stress is also important and has been associated with increases in stress hormones. Recent studies have shown increases in the urine cortisol-to-creatinine (UCC) ratio in cats housed under differing housing conditions within shelter situations. UCC were increased in cats not exhibiting outward signs of stress[44]. UCC levels were also reported to be higher in privately owned cats visiting a veterinarian's office compared to UCC levels in the same cats when at home, suggesting that cats are quite sensitive to environmental stressors[45].

The results of this study show an increased rate of mortality in FIV-infected cats, both residing in sanctuary housing (age-adjusted rate ratio 11.7) and in private homes (age-adjusted rate ratio 4.1) compared to FIV-uninfected cats. Recent studies have reported that FIV-infected cats may succumb to illnesses unrelated to FIV infection and that the lifespan of an FIV-infected cat is not statistically different from an FIV-uninfected cat[1,4,46]. While a few FIV-infected cats in this study died of causes unrelated to FIV infection, such as trauma and urethral obstruction, the majority (86%) of FIV-infected cats in this study had necropsy findings consistent with FIV infection without other differential diagnostic findings for cause of death. Importantly, in this study, FIV-infected cats that developed clinical signs of illness had an increased mortality rate after adjustment for age and environment. These results support an association between clinical signs of illness on physical examination and progression to early mortality, however, as previously reported in the literature the time to death following onset of clinical signs is variable between FIV-infected cats. In studies of naturally acquired infection, the time to death, following observation of clinical signs of illness, has ranged from 1 month to more than 41 months[47].

The choice of CBC and chemistry parameters evaluated in this study was made based on evidence that FIV causes chronic inflammation, immunosuppression, chronic disease and depletion of lymphoid cells, particularly in the mucosal lymphoid tissue[28,29,48-53].

The association of a greater decline in HCT and ALB with mortality may be explained by chronic inflammation associated with FIV infection, although inflammation associated with concurrent infections or neoplasia may also play a role. Increased circulating pro-inflammatory cytokines, interleukin-1β (IL-1β), IL-6 and tumor necrosis-alpha (TNF-α), cause production of positive acute phase proteins in the liver; at the same time the inflammatory cytokines interfere with production of negative acute phase proteins such as ALB[54,55]. This loss of ALB during chronic FIV infection may be associated with higher levels of circulating pro-inflammatory cytokines that have been reported in cats in the symptomatic stage of FIV infection compared to those in the asymptomatic stage of infection[50]. It may also be that ALB was lost via the kidneys or gastrointestinal tract (GIT) but direct conclusions cannot be drawn from this study. Renal values were not assessed for their association with illness or mortality in this study and urine protein-to-creatinine ratio was not performed in these cats. TP, GLOB, CHOL and GLU were not associated with illness or mortality suggesting that gastrointestinal loss of ALB was not an issue but intestinal biopsies and intestinal fatty acid binding protein levels were not performed to assess GIT integrity. In terms of application, a further 10% decrease in ALB in FIV-infected cats would correlate with a 3.3 fold increase in mortality rate over a 1-year interval and 2.2 fold increase over a 6-month interval ($e^{(-10*-0.119)}$ and $e^{(-10*-0.079)}$, respectively). The association of a greater decrease in HCT with mortality rate might be explained by anemia of chronic disease. This process has been reported to be associated with chronic inflammatory conditions and neoplastic disease, such as lymphoma, and may result in reduced erythroid production or iron deficiency associated shortening of the red blood cell life span.

In FIV-infected cats, a greater decrease in the percent change of CD4 was associated with an increased illness rate over the 1-year and 6-month time intervals. CD4 T cells are a driving force of the adaptive immune system and play a key role in orchestrating the humoral and cell-mediated responses to infection. The progressive loss of CD4 T cells in FIV-infected cats during the acute and asymptomatic stages of infection compared to FIV-uninfected cats has been widely reported; CD4 T cell counts in the end stage of FIV infection are not often reported. While CD4 T cell counts of <21 cells/mL in FIV-infected cats with end stage disease have been reported; it has also been reported that FIV-infected cats have tolerated low (<50 cells/mL) CD4 T cell levels

without showing outward signs of clinical illness during the asymptomatic stage of infection. Although the results of this study show an association between CD4 T cell percentage and illness rate, the low hazard rate ratio suggests that a greater change in CD4 T cells would not be sufficient as a single tool to indicate progression to disease. A greater decrease of percent change in CD4 T cells in cats in this study had limited practical application. For example, a further 50% decrease in CD4 T cells would only result in a 1.9 fold increase in illness over a 1–year interval and 1.7 fold increase in illness over a 6-month interval ($e^{(-50*-0.013)}$ and $e^{(-50*-0.011)}$, respectively).

A greater increase in the CD4:CD8 ratio over a 1-year interval and NEUT over a 6-month interval was associated with an increased illness rate. Goto et al[14] reported an increase in the CD4:CD8 ratio for cats in the end stage of disease compared to those in the chronic stage of disease. This change in ratio was attributed to loss of both CD4 and CD8 cells in the end stage of disease because the end stage ratio was still markedly lower than that of the asymptomatic cats. It is difficult to determine why a greater increase in the CD4:CD8 ratio was associated with illness in these study cats. It is possible that the timing of illness was early enough in the disease course that a sufficient number of CD4 T cells were still present in relation to CD8 T cells and kept the CD4:CD8 ratio at a higher level. Conversely, it may be that expansion of $CD\beta^{low}$ cells within the CD8 T cell population was not at a point great enough to decrease the CD4:CD8 ratio in comparison to the CD4 T cell population. Due to the fact that these infections were naturally acquired without a known infection date, it is impossible to know the duration of infection and how this may have impacted immunosuppression and T cell levels. The association of a greater increase in NEUT with illness may reflect a normal fluctuation of cell counts in this small group of FIV-infected cats but it is also possible that the immune response mounted by the FIV-infected cats was still adequate at this stage in chronic disease.

There are several limitations to this study that may have affected our results. Survival analysis was used to statistically account for loss to follow-up in the different groups of cats. However, an assumption that the reasons cats were lost to follow-up were unrelated to mortality and illness cannot be validated. Our use of a stepwise procedure to identify significant associations between markers and outcomes of interest is considered exploratory and a confirmatory study is needed. The cats enrolled in this study were all naturally infected with FIV and the exact date of infection, the infecting dose at the time of infection and duration of infection are unknown. Given these unknowns direct longitudinal comparisons among the cats was not possible. Additionally, as

these cats were all sourced from shelters, the age assigned at the time of shelter admission, and that used for the age at enrollment, was an estimate. Given the long term chronic nature of FIV infection the time frame of the study (up to five years) might not have been long enough to realize the natural disease progress of FIV-infected cats.

4.5 Conclusion

Cats infected with FIV had a higher incidence rate of mortality and illness compared to FIV-uninfected cats and those that residing in sanctuary housing had a higher incidence rate of mortality and illness compared to FIV-infected cats in private homes with <10 cats. Percent change in inflammatory markers ALB and NEUT and lymphocyte immunophenotype were associated with an increased rate to illness or death in FIV-infected cats, however, the incremental changes and associations are considered exploratory in this small group of cats and cannot be extrapolated to FIV-infected cats in general. Furthermore, the change in rate ratio for lymphocyte immunophenotype values was small and may not be practically important as a single monitoring tool for illness and mortality. These results do support the hypothesis that inflammation and immune activity are important components associated with FIV disease progression. The results also highlight the importance of housing environment on illness and mortality in FIV-infected cats, which is key in defining best management practices as the animal sheltering community more commonly seeks to place FIV-infected cats for adoption. Further investigation into the interaction between inflammatory pathways, immunophenotype changes and housing environment in cats naturally infected with FIV is warranted.

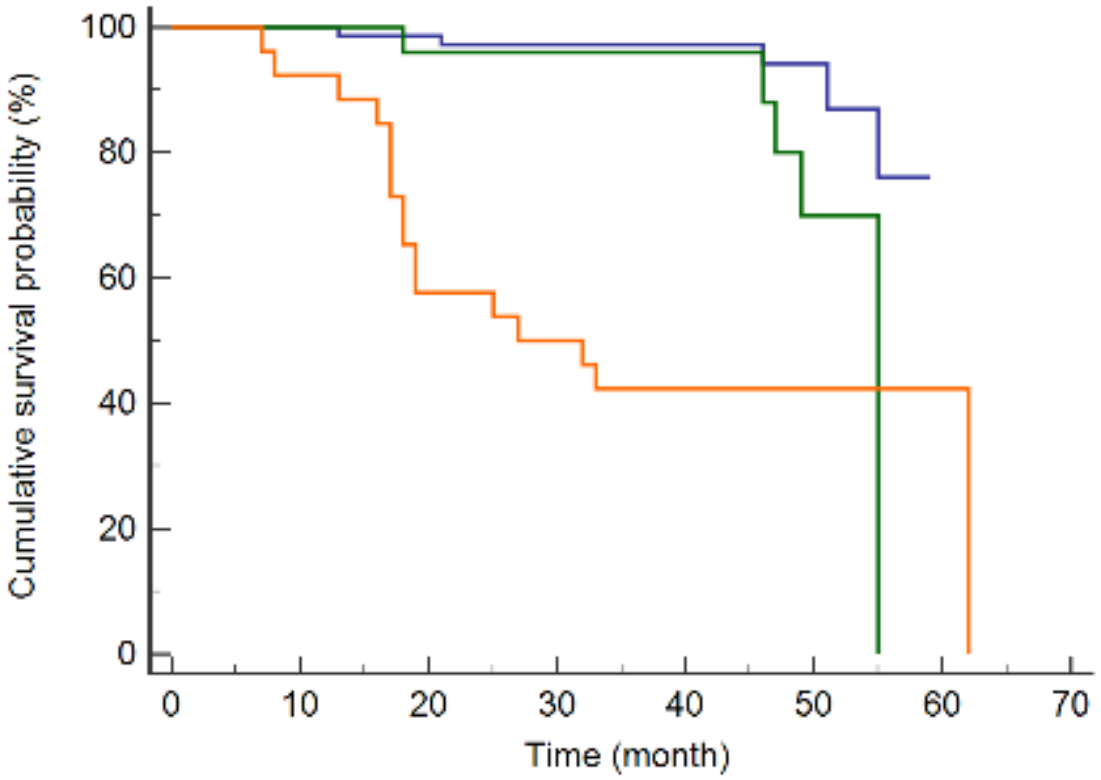

Figure 4.1. Survival curves for Mortality in FIV-infected and FIV-uninfected cats

Blue line: FIV -; Green line: FIV+ in private home; Orange line: FIV+ in sanctuary

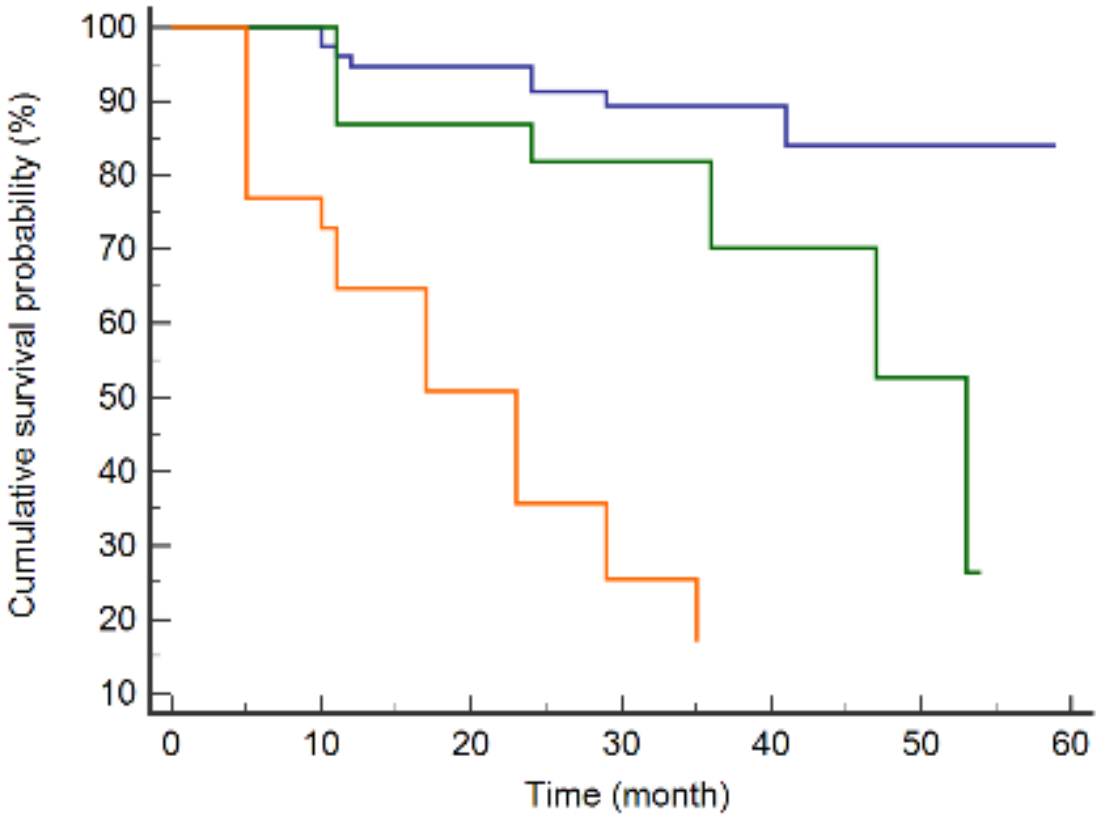

Figure 4.2. Survival curves for Illness in FIV-infected and FIV-uninfected cats

Blue line: FIV -; Green line: FIV+ in private home; Orange line: FIV+ in sanctuary

Table 4.1. Age and Sex of FIV-infected and FIV-uninfected cats

[a] All cats were previously spayed or neutered at time of enrollment

[b] Median age at enrollment for FIV-infected and FIV-uninfected cats

	Groups		
	FIV-negative n = 78	FIV-positive sanctuary housing n = 26	FIV-positive private homes n = 26
Males (%)[a]	50 (64)	19 (73)	18 (69)
Females (%)[a]	28 (35)	7 (27)	8 (31)
Age (years) at enrollment[b] (range)	5 (1.5-11)	3 (1.5-10)	4.5 (2-9)

Table 4.2. Analytes that are significantly associated with mortality and illness in FIV-infected cats investigated in Cox regression with a stepwise procedure

[a] A negative coefficient indicates that a greater decrease in the marker prior to death/illness is associated with an increase in the rate of mortality and illness. A positive coefficient indicates that a greater increase in the marker prior to death/illness is associated with an increase in the rate of mortality and illness.

Mortality	Variable	Coefficient[a]	P-value
1 year interval in FIV+ cats	TP	0.072	0.042
	ALB	-0.119	<0.001
6 month interval in FIV+ cats	HCT	-0.049	0.001
	ALB	-0.079	0.004
Illness	Variable	Coefficient[a]	P-value
1 year interval in FIV+ cats	CD4	-0.02	0.002
	CD4:CD8 ratio	0.009	0.015
	ALB	-0.043	0.039
6 month interval in FIV+ cats	CD4	-0.011	0.041
	NEUT	0.009	0.04

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CHAPTER 5. CONCLUSION

Research into FIV infection is complicated due to the lengthy chronic asymptomatic phase of disease, inability to identify cats that will progress to end stage disease, lack of factors associated with disease progression and expense associated with long term monitoring. To date, the majority of longitudinal research has focused on the acute and early chronic stages of infection in experimental models of FIV infection[1-4]. Research investigating the transition from late chronic to end stage infection is sparse; published findings are based primarily on the outcome information of only a few cats with natural infection which makes extrapolation to the general FIV-infected population difficult[5-7].

This research project followed a cohort of cats naturally infected with FIV and uninfected control cats over a period of up to 5 years. Comparisons were made between FIV-infected cats and FIV-uninfected cats and between FIV-infected cats in sanctuary housing and FIV-infected cats in private homes of <7 cats. The purpose of this study was to determine temporal patterns in routine laboratory parameters and lymphocyte immunophenotype, describe the illness and mortality rates, and assess the association of inflammatory, lymphocyte immunophenotype and hepatic markers with incidence of illness and mortality.

The most striking finding of this study was the disparity in hazard rates for illness and mortality among the groups of cats. Compared to FIV-uninfected cats, mortality rate was increased 11.7 fold in FIV-infected cats in sanctuary housing and 4.1 fold in FIV-infected cats in private homes. The mortality rate was increased 5.1 fold for FIV-infected cats in sanctuary housing compared to FIV-infected cats in private homes. Compared to FIV-uninfected cats, the illness rate was increased 15.1fold for FIV-infected cats in sanctuary housing and 3.0 fold for FIV-infected cats in private homes. Furthermore, FIV-infected cats that became ill had a 3.8 fold increase in mortality rate compared to FIV-infected cats that did not become ill. These findings support an association

between clinical signs of illness and early mortality, however, time to death following the onset of clinical signs was variable and similar to the 1 month to greater than 41 months that has been previously published[5]. Direct comparison of illness and mortality rates between FIV-infected cats living in different environments has not been previously reported. These results were surprising, although not completely unexpected. Determination of causes for increased illness and mortality rates was beyond the scope of this study, however, it has been reported that cats living in shelter housing have a high prevalence of illness; particularly upper respiratory infections[8-11]. Increased incidence and prevalence of upper respiratory infections in shelters have been associated with increased numbers of cats within the shelter, ineffective cleaning and disinfecting protocols, lack of standard vaccination protocols, presence of upper respiratory disease within the shelter, and lack of quarantine and isolation procedures for cats entering a facility[8-11]. Several studies have reported higher urine cortisol-to-creatinine (UCC) levels in cats under certain shelter housing conditions, and in cats at a veterinary facility compared to UCC levels in the same cats at home which suggests that cats are sensitive to environmental conditions[8,12,13].

Differences in temporal patterns for CD4 and total CD8 T cells and lymphocyte counts showed a greater loss in FIV-infected cats compared to uninfected cats. CD4 T cells are the primary target of FIV infection but CD8 T cells can also be infected and progressive loss of T cells through cell mediated, apoptotic or anergic mechanisms have been reported[14-16]. Although anticipated, differences in temporal patterns for $CD8\beta^{low}$ cell counts were not seen, despite $CD8\beta^{low}$ cell counts being consistently higher in FIV-infected cats. It is possible that maximal expansion of $CD8\beta^{low}$ cells had already occurred in the FIV-infected cats. This may have impacted the CD4:CD8 ratios, as they were lower in FIV-infected cats, as expected, but no difference in temporal pattern was seen.

Notable differences in temporal patterns between FIV-infected cats in sanctuary housing and cats in private homes were seen for absolute white blood cell counts and total protein values. White blood cell counts and total protein levels increased in FIV-infected cats in sanctuary housing compared to decreased values in FIV-infected cats in private homes. Median total protein levels for both FIV-infected groups were above the reference interval throughout the study. The increased total protein levels may reflect hypergammaglobulinemia thought to be secondary to B cell stimulation in FIV-infected cats. The sanctuary housed FIV-infected cats had consistently higher median globulin values and absolute neutrophil counts, and lower median albumin values compared to cats in private homes, although differences in temporal patterns were not seen. These

findings may reflect the increased illness rate in sanctuary housed cats and suggest an increased inflammatory process that may be related to increased illness or stress within the environment.

Associations between inflammatory and lymphocyte immunophenotype markers and increased illness and mortality rates were found in FIV-infected cats. This lends further support to previously published findings linking chronic inflammation and alterations in T cells with chronic FIV infection[2,3,17-19]. A greater decrease in CD4 T cell and albumin percentages and greater increase in CD4:CD8 ratio and neutrophil percentages were associated with increased illness rate. Increase in mortality rate was associated with a greater increase in total protein and greater decrease in albumin and hematocrit percentages. However, the individual hazard rate associated with each marker was weak. Inflammation in chronic disease states has been associated with albumin loss. This is thought to be secondary to increased pro-inflammatory cytokine and positive acute phase protein production and decreased negative acute phase protein production, such as albumin[20,21]. FIV-infected cats are reported to have increased circulating pro-inflammatory cytokines and this may be a potential cause of the association between decreased albumin and increased illness and mortality[19]. Loss of CD4 T cells following FIV infection is well documented and association between decreased CD4 T cells with illness and mortality was expected, however, the associations were anticipated to be much stronger. This supports previous postulations that cats can tolerate low CD4 T cell levels without developing disease. Previously published studies have reported conflicting observations between clinical signs of illness and low CD4 T cell counts in FIV-infected cats[7,22].

Overall, this study showed an increased risk of illness and death in FIV-infected cats compared to uninfected cats. Furthermore, it uncovered a discrepancy in illness and mortality rates, not previously reported in the literature, between FIV-infected cats living in sanctuary housing and those living in private homes. This raises the question of whether environment plays a role in illness and mortality associated with FIV infection. Further investigation into the role of environment in FIV disease progression is warranted; particularly as animal shelters increasingly seek to adopt rather than euthanize FIV-infected cats. The associations between inflammatory and lymphocyte immunophenotype markers and illness and mortality rates were considered exploratory and extrapolation to the general population of FIV-infected cats cannot be made; especially as associations were weak. However, given the lack of readily available monitoring techniques to assess disease progression in FIV-infected cats, further investigation into these health parameters may be necessary. Ultimately, new, reliable and easily performed diagnostics

are needed to better assess the immune response and inflammation associated with long term, chronic FIV infection. Better tools to assess immune health in relation to the progression of FIV infection will lead to better management techniques and earlier medical intervention to preserve and extend length and quality of life.

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